EVALUATION OF PLATELET ACTIVATION BY ESTIMATING SERUM sP-SELECTIN LEVEL AND MORPHOLOGICAL CHANGES OF PLATELETS IN PERIODONTAL HEALTH AND DISEASE.

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CERTIFICATE

This is to certify that **Dr.P.RAMESH**, Post graduate student (2008 – 2011) in the Department of Periodontology, Tamilnadu Government Dental College and Hospital, Chennai – 600 003 has done this dissertation titled **"EVALUATION OF PLATELET ACTIVATION BY ESTIMATING SERUM sP-SELECTIN LEVEL AND MORPHOLOGICAL CHANGES OF PLATELETS IN PERIODONTAL HEALTH AND DISEASE."** under our direct guidance and supervision in partial fulfillment of the regulations laid down by **The Tamil Nadu Dr.M.G.R. Medical University**, Chennai -600 032 for **M.D.S.,** (Branch – II) **Periodontology** degree examination.

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ABSTRACT

Background: The association of periodontitis and cardiovascular diseases is a long standing dilemma and the underlying mechanism between these two diseases is not clear. Regular transient bacteremia occurs during tooth brushing and mastication in periodontitis patients, which provoke the synthesis of proinflammatory mediators in systemic circulation leading to systemic inflammation and platelet activation. Consequently coagulation cascade activation leads to atherothrombosis and CVD.

Aim: This study is aimed to investigate whether periodontitis patients have higher platelet activation compared to controls.

Materials and Methods: About 80 subjects were included and categorized into control group (n=40) and study group (n=40). This study comprises of a qualitative and a quantitative analysis. In qualitative analysis change in shape and aggregation pattern of platelets were assessed by spreading analysis test using light microscopy and serum sP-selectin level is estimated quantitatively by ELISA method.

Results : In present study patients with periodontitis showed significantly higher number of activated platelets and pathological aggregation pattern with elevated serum sP-selectin level when compared to controls and is positively correlated with the disease severity.

Conclusion: Platelet activation is associated with periodontitis and it is severity dependent. Chronic persistent low grade systemic inflammation due to periodontitis may contribute to cardiovascular disease development and progression. Chronic periodontitis could be a possible risk factor for cardiovascular and cerebro-vascular diseases.

DECLARATION

TITLE OF DISSERTATION	**"Evaluation of Platelet activation by estimating serum sP-selectin level and morphological changes of platelets in periodontal health and disease."**
PLACE OF STUDY	Tamil Nadu Government Dental College & Hospital, Chennai-600003
DURATION OF THE COURSE	3 Years
NAME OF THE GUIDE	Dr. R. MAHEASWARI
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I hereby declare that no part of the dissertation will be utilized for gaining financial assistance/any promotion without obtaining prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai-600003. In addition, I declare that no part of this work will be published either in print or in electronic media without the guide who has been actively involved in dissertation. The author has the right to reserve for publish of work solely with the prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai-600003.

Head of the Department **Guide** **Signature of the candidate**

CONTENTS

LIST OF ABBREVIATIONS

AAP	–	American Academy of Periodontology
Agg+	_	Aggregation
CAL	–	Clinical attachment level
CEJ	_	Cemento – Enamel Junction
cPRP	_	Citrated platelet rich plasma
CHD	_	Coronary Heart diseases
CVD	_	Cardiovascular diseases
ELISA	–	Enzyme linked immune sorbent assay
(IL)-1β	_	Interleukin 1 Beta
IL-6	_	Interleukin 6
MI	_	Myocardial Infarction
PAAP	_	Platelet aggregation-associated protein
PAT I	_	Platelet Aggregation Test I
PAT III	_	Platelet Aggregation Test III
P. Gingivalis	_	Porphyromonas gingivalis
PLT	_	Platelet
PPD	_	Probing pocket depth
PAR-1 & 4	_	Protease-activated receptors 1 & 4
sP - Selectin	_	Soluble P-Selectin
vWF	_	Von Willibrand factor

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INTRODUCTION

Periodontitis is an inflammatory disease of the supportive tissues of the teeth, characterized by gradual loss of tooth supporting alveolar bone, and affects up to 10% of the population in its most severe form ***(Pihlstrom BL 2005)***[91] . The primary etiologic factor of periodontitis is the subgingival infection with a group of Gram-negative pathogens. The major bacterial species associated with periodontitis are *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) ***(van Winkelhoff AJ, 2002)***[107] **.** Inflammation in the periodontal tissues results in areas of ulceration of the epithelium in the periodontal pocket, which leads to dissemination of oral bacteria into the circulation during mastication ***(Geerts SO,2002)***[39]**.** Transient bacteremia in periodontitis patients underlie chronic production and systemic increases of various proinflammatory mediators, including Interleukin (IL)-1β, IL-6, C-reactive protein and Tumor-necrosis factor (TNF)- α. ***(Loos BG 2005*** [67]***; Paraskevas S,2008)***[8 9]

Systemic inflammation can cause increase in the number of platelets and platelet activation ***(Klinger MH,2002)*** [63]**.** Activation of platelets leads to the release of pro-inflammatory mediators and exposure of pro-inflammatory receptors, resulting in platelets binding to leukocytes and endothelial cells. These functions make platelets essential participants in both thrombotic and inflammatory reactions across the vasculature. ***(Weyrich AS,2004)*** [113]

Platelet activation comprises a change in platelet shape, platelet aggregation and the release of platelet constituents. Platelet shape change is an early event in the activation of platelets. When platelets adhere to the subendothelial matrix, they

change their shape from discoid to spherical with the extrusion of the pseudopods.***(White JG 1974)*** [114]

Also studies have demonstrated that periodontal pathogen like *P.gingivalis* can activate blood platelets and induce platelet aggregation through hemagglutinin domain protein HgP_{44}.***(Naito 2006)*** [82]

P- selectin is a member of selectin family of cell surface receptor which is located in the membrane of the secretory granules (alpha granules) of platelets and in the membrane of the Weibel–Palade bodies of the vascular endothelial cells**(*McEver RP, 1990)*** [72] **.** P selectin redistributes from the membrane of the granules to the plasma membrane when platelets and endothelial cells are activated and thus degranulated. ***(Johnston GI,1989)*** [58]

Platelet activation has been implicated in the development of atherosclerosis, atherothrombosis and subsequent coronary vascular and cerebrovascular diseases ***(Cahill MR,1993)*** [22] **.** Abnormal platelet activation has also been associated with deep vein thrombosis **(*De Boer AC,1981)*** [30]**,** inflammatory bowel disorders ***(Collins CE, 1994)*** [25] , cancer, peripheral vascular disease, Alzheimer disease ***(Sevush S,1998)*** [101], and atrial fibrillation ***(Minamino T,1999)*** [78]

Epidemiological and intervention studies have associated periodontitis with atherosclerosis and cardiovascular diseases (CVD). The underlying mechanisms of this relationship are still obscure ***(Tonetti MS,2007)*** [106].

Since only very few studies have been documented regarding association of periodontitis and platelet activation , the present study was undertaken to evaluate whether periodontitis patients have higher state of platelet activation by estimating the serum sP-selectin expression and platelet morphological changes and aggregation pattern compared to healthy controls.

AIM AND OBJECTIVES

The aim of the present study was to investigate whether periodontitis patients have a higher state of platelet activation compared to healthy controls.

For this purpose, the following objectives were undertaken:

1) To estimate soluble- P selectin level in control and patients with chronic periodontitis by ELISA method.

2) To compare morphological changes and aggregation of platelets by spreading analysis method using light microscope in control and patients with chronic periodontitis .

REVIEW OF LITERATURE

Overview of platelets

The normal platelet count is in the range 150–350 x 10^9/ litre and the platelet has a life span of approximately 8–10 days **(*Bautista AP,1984)* [8]** . Resting platelets are discoid and have a smooth, rippled surface ***(Felder R,1985)* [34]** with a diameter which averages 1–2 μm and a mean cell volume of around 5–6 fl. Platelets are derived from the megakaryocytes in the bone marrow. These megakaryocytes arise by a process of differentiation from the haemopoietic stem cell and undergo fragmentation of their cytoplasm to produce platelets ***(Avraham H,1993)* [5]**

The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to the vessel wall injury, following platelet activation. The platelet surface has various receptors and there are diverse stimuli, which activate platelets, with equally diverse platelet responses to these stimuli, mediated by the binding of various stimulants to specific platelet receptors.

It is possible to categorize platelet responses into the 'reversible' platelet responses, which include adhesion, shape change and reversible aggregation and, the 'irreversible' platelet responses that comprise release reaction and secondary irreversible aggregation ***(Seiss W,1989)* [100].** Platelet adhesion and a change in shape are the initial physiological responses towards the development of haemostatic plug or thrombus ***(Sixma JJ,1991)* [104].**

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PLATELET ADHESION

One of the earliest events following blood vessel injury is the adhesion of platelets to the areas denuded of endothelium***(Seiss W.1989)*** [100]. A number of unique features differentiate platelet adhesion from other adhesive processes. Platelets bind to a variety of extracellular matrix connective tissue components, such as fibrin, collagen and laminin ***(Nurden AT,1993)*** [83]**.**

Platelets also seem to adhere to particulate matter in the blood stream, bacteria and other microorganisms, macrophages and the artificial surfaces of prosthetic devices ***(Packham MA, 1984)*** [87]**.** This process of platelet adhesion may play a fundamentally important role in atherogenesis.

To provide adequate haemostasis in an injured vessel, platelet adhesion leads on to platelet aggregation; thus, activated platelets that are involved in adhesion result in further interaction with inactivated platelets in the blood stream. High concentrations of integrins and other glycoproteins on the platelet membrane play an important role in platelet adhesion to the extracellular matrix. Indeed, the adhesion of platelets to the sub-endothelium under flow conditions is well recognized to be mediated by a number of proteins in the plasma, of which the more important ones are vWF, fibronectin, fibrinogen and thrombospondin ***(Packham MA,1984)*** [87]**.**

PLATELET SHAPE CHANGE

When platelets adhere to the sub-endothelial matrix, they change their shape from discoid to spherical with the extrusion of the pseudopods ***(White JG. 1974)*** [114] . There is a suggestion that the procoagulant activity and subsequent production of

thrombin increases on the platelet surface due to this platelet shape change ***(Ehrman M,1978)*** [32].

Platelet shape change is influenced by a variety of factors. For example, it is known that platelets could activate and change shape in vitro after venepuncture ***.(Bamber E,1978)*** [6]**,** following contact with different substances, and by exposure to varying temperatures ***(Watts SE,1986)*** [112]**.**

Nachmlas VT 1983 [77] found that most stimuli cause a change in shape .This change involves first the formation of very fine (0.1 μm diameter) pseudopodia (i.e., filopodia) from the rim of the disc, followed by a general "rounding up" of the platelet so that it becomes a spiny sphere, often with much broader pseudopodia.. Importantly, a reliable method of differentiating in vivo from in vitro platelet shape change is not available at the moment.

PLATELET AGGREGATION

The linking of the platelets via fibrinogen brings about platelet aggregation. A more number of agents (including ADP, epinephrine, collagen and thrombin) can induce platelet aggregation ***(Kinlough-Rathbone RL,1977)*** [62]. Simplistically, vWF and fibrinogen bind to receptors on one platelet and crosslinks to the other platelet by binding on to receptors on the latter ***(McManama G,1986)*** [73] **.**

'Reversible' platelet aggregation is induced by low concentrations of platelet stimuli in the presence of extracellular Ca2+ and/or Mg2+, whereas high concentrations of the agonists can cause 'irreversible' reaction. The latter is the result of the platelet release reaction, which relates to the release of arachidonic acid metabolites ***(Hamberg M, 1974)*** [42] , especially endoperoxides and thromboxanes, and the secretion of platelet constituents from the dense granules (ADP, ATP,

serotonin, Ca2+), alpha granules (beta thromboglobulin, Platelet Factor 4, platelet derived growth factor etc.) and from the lysosomes ***(Holmsen H,1969)*** [48].

. The release reaction, which augments the platelet aggregation, is regulated by two positive feedback loops. Firstly, the endoperoxides, thromboxane A2 and ADP, which are released during the reaction cause further expression of the fibrinogen receptors on the platelet surface by intracellular mechanisms, thus inducing further platelet aggregation ***(Holmsen 1977)*** [49].

Secondly, the synergism between the different platelet agonists augments platelet aggregation ***(Altman R,1986)*** [2.] Full platelet aggregation can also be induced by the simultaneous addition of sub-threshold levels of platelet stimuli, which fail to induce platelet aggregation on their own merit. Thus, the synergistic action of the primary platelet stimulus, other sub-threshold agonist stimuli and the products of platelet release reaction build up an efficient 'multi-stimulus' for platelet aggregation. Increased intra-platelet levels of cyclic nucleotides also inhibit platelet aggregation ***(Sakuma I, 1990)***[95] . These activated platelets contribute to haemostasis and it is widely believed that these activities are relevant to thrombosis, especially to arterial thrombosis, where the bulk of the occlusion often seems to be due to platelet mass.

Studies on platelet aggregation

Born 1962 [12] observed that ADP induced aggregation of PLT could be demonstrated in citrated platelet rich plasma (cPRP) . This kind of PLT aggregation test (PAT I; ***Breddin 1965***) [14,] defining different grades of PLT activation microscopically, was modified later by ***Breddin in 1976***[15] (PAT III) using cPRP for the performance of a photometrical assay (***Breddin 1977***) [17]. Spontaneous Platelet

aggregation (PAT III) was recorded on a universal aggregometer (Braun, Melsungen) using an Eppendorf photometer "1001" by Netheler & Hinz, Hamburg.

Salzman (1963)[96] carried out microscopic investigation of the filter used in his study showed extensive PLT aggregation on the glass beads. It was concluded that the platelet retention by such filters could be a measure of platelet aggregation and adhesion.

Platelet aggregation due to Ca^{++} dependent processes was also related to Platelet activation by preanalytical circumstances (e.g., time from withdrawal of blood to the performance of the assay) **(*Breddin 1974* [16]*, Bauer 1980*).**[7]

Breddin 1974[16] concluded that Platelet aggregation was also attributed to inflammatory and atherosclerotic processes . An increased aggregation may be interpreted as a thrombophilic risk variable as demonstrated for either patients with arterial diseases as well as for patients with venous thrombotic diseases.

Platelet aggregation has been studied by monitoring the changes in the optical density of cPRP for PAT III . This assay has been routinely used to evaluate hyperactivation of Platelet and can be attributed either to arterial or venous vascular diseases.(***Breddin 1976***[15] ***and 1999)***[20]

Breddin 1985[19] **,** ***1986*** [18] observed PAT III has been proved to be of predictive value for new vascular occlusions in diabetics . After the development of PAT I ***(Breddin 1965)*** [14]**,** several approaches were developed to measure Platelet aggregation other than PAT III e.g., the Wu & Hoak test (***Wu 1974*** [117]***, Velaskar 1982)***[108] using flow cytometric analysis which was of limited value since it was not standardized .

Mc Pherson 1987 [74] , a vicious circle was hypothesized by which Platelet that get into contact with foreign surfaces are activated (or even damaged), express

bioactive amines, stimulating further Platelet and leading to aggregation and at last thrombus formation.

Studies on Platelet aggregation associated with Periodontitis

Herzberg MC 1983 [45] stated that *Streptococcus sanguis* is the most prevalent species in dental plaque and frequently identified in the polymicrobial bacteremias from dental foci. Platelet aggregating strains (Agg+) of *S. sanguis* induce aggregation of human platelets in vitro. This phenomenon is mediated by the expression of the *platelet aggregation-associated protein (PAAP)* on the surface of certain strains.

In ***1989, Mattila KJ*** [70] stated that dental infections may also contribute to the risk of atherosclerosis and MI.

Gingipain-K from *P. gingivalis* interferes with the blood-clotting system by human platelet aggregation. *P. gingivalis* cleaving both fibrinogen and kininogen to release the vasoactive peptide, bradykinin. ***(Scott CF,1992).*** [99]

Herzberg MC 1994[46] observed that *Streptococcus sanguis*, the predominant microorganism found in bacteremias following toothbrushing or dental procedures, and *P. gingivalis*, an invasive periodontal pathogen, also induce platelet aggregation

In the same year ***Steiner M et al*** [105]**,** stated that *S. sanguis* may also induce thromboembolic events. The Aggregation (Agg+) phenotype may be associated with the occurrence of disseminated intravascular coagulation- like syndrome in immune-compromised patients. Approximately 60% of human isolates express the Agg+ phenotype.

Beck 1996 [9] postulated that the association between severity of periodontal disease and the risk of CHD may be due to the biologic burden of Gram-negative infection and the inflammmatory mediators of periodontal disease.

Zambon 1997 [118] observed that atherosclerotic plaques are commonly infected with Gram-negative periodontal pathogens, including *A. actinomycetemcomitans* and *Porphyromonas gingivalis.*

Pham, 1998 [90] was first to demonstrate that the membrane vesicles were also potent activators of human platelets, and that purified gingipain-R from the membrane vesicles caused this activity. This is an important finding in that it lends support to the concept that shedding of these membrane vesicles may be an important virulence factor not only in inflammation but also in micro thrombus formation in humans.

Herzberg MC1994 [46] hypothesized that Agg+ S. sanguis is thrombogenic; experimental bacteremias in rabbits would cause thrombosis and acute changes in cardiopulmonary function. Intravenous infusion of 4 X 10s colony forming units (CFUs) of S. sanguis 133-79 (Agg+) causes a rise in blood pressure within the first minute. Infusion of an Agg" strain or saline was without effect. The Agg+ strain caused dose dependent changes in blood pressure, heart rate, and the percentage of rabbits showing increased cardiac contractility.

Sharma et al 2000 [102] have shown that *P. gingivalis* membrane vesicles aggregate murine platelets.

Lourbakos et al.2001 [69] reported that two forms of purified gingipain-R, the 50- kDa RgpB and the 95-kDa RgpA, caused aggregation of human platelets with efficiency comparable with thrombin, by a mechanism involving cleavage of the

protease-activated receptors (PAR)-1 and –4, expressed on the surface of platelets. purified gingipain-R which aggregated platelets at 0.01 mg/ml or 0.1 nM.

Li et al.2002 [65] reported that mouse model of atherosclerosis using apolipoprotein E-deficient mice, intravenous and oral inoculations of *P. gingivalis* cells were found to accelerate atherogenic plaque progression and to enhance vascular activation ***(Lalla et al., 2003***) [64] respectively.

P-SELECTIN: Structure and function

P-selectin (CD62P, previously also known as GMP- 140 and PADGEM) is, alongside E-selectin (CD62E) and L-selectin (CD62L), a member of the selectin family of adhesion molecules, sharing common structural features but having different tissue distributions. It has a mass of approximately 140 kDa and is a protein rich in cysteine, containing several complex *N*-linked oligosaccharides chains. The extracellular domain is arranged as a lectin region (the amino-terminal of 120 amino acid residues), an epidermal growth factor-like region and nine discrete complement regulatory-like regions (each about 60 amino acid residues in length). In addition, there is a cross-membrane segment and an intra-cytoplasmic tail .

P-selectin is so named because it is a component of the cell membrane of the α granules of platelets, the organelle which stores coagulation- related substances such as β- thromboglobulin and platelet factor 4. It is also a component of the membrane of the Weibel-Palade body (the endothelial cell specific organelle which stores von Willebrand factor) and is rapidly mobilised to the cell surface by various stimuli including inflammatory mediators ***(Bevilacqva 1993*** [10] ***& Wagner 1993)***[110]

The mucin-like glycoprotein ligand for P-selectin (PSGL-1) bears *O*- and *N*-linked carbohydrates, and is found on neutrophils, Natural Killer cells, monocytes and

T-lymphocytes, giving rise to the hypothesis that it is involved in an interaction between these leukocytes and the endothelium, (possibly as part of the rolling: binding:activation sequence), between platelets and neutrophils, and between platelets and the endothelium***(Geng 1990)*** [40]

Hattori 1989 [44] reported that in cultured endothelial cells, membrane expression of P-selectin can be induced with inflammatory mediators such as thrombin, lipopolysaccharide, tumour necrosis factor, oxygen radicals, complement components and histamine. Therefore it may be that this increased surface expression is a further marker of activation. However, it is likely that this expression is transient and that it is endocytosed within a period of up to 4 hr, possibly to be recycled for further use. It is strongly expressed by endothelium overlying fibro-fatty and complex atherosclerotic plaques, weakly on endothelium overlying fibrous atherosclerotic plaques and is absent from normal arterial endothelium ***(Johnson - Tidey 1994).*** [57]

McEver RP 1989 [71] stated that P-selectin is a membrane constituent of both platelet α granules and endothelial cell Weibel-Palade bodies, soluble levels can be derived from either source.

Hamburger 1990 [43] found that thrombin will also induce increased surface expression of P-selectin on platelets for up to 1 hr, a finding that leads to the conclusion that increased cell membrane expression of P-selectin is a marker of stimulated or activated platelets . This may find clinical value as platelet P-selectin expression may be quantified with relative simplicity by flow cytometry .(***Shattil SJ 1987).*** [103]

The role of P-selectin could be twofold: first, it might inhibit excessive leukocyte recruitment by inhibiting leukocyte adhesion to the endothelium as was shown with purified platelet P-selectin and downregulate neutrophil activation; second, it could inhibit resting platelet rolling on P-selectin and thus prevent excessive platelet recruitment to the growing platelet thrombus.(***Gamble JR,1990)***[38]

Dunlop LC 1992 [31] stated that soluble P-selectin is found at concentrations of about 0.1 to 0.2 µg/mL in human plasma.

Increased levels of soluble P-selectin have been described in diseases such as ischemic stroke, atherosclerosis, hypertension, thrombotic thrombopenic purpura, eclampsia, thromboembolic diseases, malaria, and diabetes as well as in different stages during the menstrual cycle of healthy women.(***Katayama M 1993).***[60]

Chong BH,1994 [24] concluded that failure of levels of soluble P-selectin to correlate with an established marker of platelet activity, *β*- thromboglobulin, implies that it is related to a different aspect of platelet activation or physiology.

Michelson AD,1999 [77] concluded that failure of levels of soluble P-selectin to correlate with an established endothelial cell marker (von Willebrand factor) , implies that soluble P-selectin is derived from platelets. This is supported by preliminary flow cytometry experiments in non-human primates which indicate that a loss of surface membrane P-selectin from platelets is accompanied by an increase in soluble P-selectin .

Administration of desmopressin (an agent which will induce the degranulation of the Weibel- Palade body) to healthy males increases levels of plasma von Willebrand factor but does not influence levels of soluble P-selectin . These data suggest that it is likely that soluble P-selectin is not the product of the endothelium. (***Jilma B,1996)*** [55]

Blann AD,1997 [11] compared of levels of circulating protein markers of endothelial cell or platelet activation with that of soluble P-selectin have suggested that activated platelets were the major source of the excess of soluble P-selectin and hypothesized that soluble P-selectin has potential as a new marker of platelet activation.

P-selectin expressed on activated platelets causes formation of platelet-neutrophil ***(Ott I,1996)***[85] and platelet-monocyte aggregates in blood***.(Rinder HM,1991)***[93] . Platelet microparticles, containing P-selectin, were also shown to support leukocyte-leukocyte interaction under flow ***(Forlow SB,2000)***[35] **.**These associations could augment infiltration of monocytes into atherosclerotic lesions,similar to L-selectin promoting secondary capture of leukocytes at sites of inflammation***.(Eriksson EE,2001)***[33]

Ikeda H, 1999 [52] have demonstrated by immunohistochemical analysis the incorporation of platelets with upregulated P-selectin to leukocytes in large arterial thrombi of a canine model of acute coronary syndromes . Also hypothesized that P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet-leukocytes aggregates.

Platelet derived microparticles associated with PSGL-1–expressing hematopoietic stem cells and facilitate their homing to the bone marrow. By a similar mechanism, P-selectin on microparticles could coat monocytes and contribute to their recruitment into atherosclerotic lesions. Interestingly, monocytoid cells were shown to roll and adhere to endothelium in vitro at a much higher shear rate when complexed with activated platelets than alone. This could help homing of the monocytes in unfavorable shear stress conditions.(***Janowska-Wieczorek A, 2001)*** [54]

Platelets and platelet microparticles were shown to deposit chemokines, such as RANTES *(**Von Hundelshausen P, 2001)*[109] or other agents,on endothelium, which can attract and activate monocytes, thus possibly promoting their recruitment into the atherotic lesions. Platelets or substances released from activated platelets were shown to induce in vitro macrophage foam cell formation and thereby could contribute to lesion growth*.(Curtiss LK,1987)*[28]

Molenaar TJM. 2003 [79] quantitated the expression of endothelial P-selectin levels in apoE_/_ mice by real-time PCR, and compared its tissue specific expression with that of VCAM-1, CAM-1 and E-selectin. Results showed P-selectin mRNA expression increases 14 fold induction was observed as compared with control mice suggesting that P-selectin is a good candidate for intervention, progression of lesion formation, lesion imaging, or targeting strategies. Furthermore, selective P-selectin antagonists by using phage display technology, such ligands may be useful in the future to identify, target or antagonize P-selectin during atherosclerosis.

Papapanagiotou 2009 [88] compared P-selectin expression in control and periodontitis patients and concluded that periodontitis is associated with increased platelet activation and via platelet activation, periodontitis may constitute a risk factor for CVD.

Assinger.A 2010 [4] study indicated that stimulation with periodontopathogens results in surface expression of P-selectin in both platelets and endothelial cells within minutes. Therefore, both cell types seem to be responsible for the observed increased plasma levels of soluble P-selectin in periodontitis.

MATERIALS AND METHODS

STUDY DESIGN AND SUBJECT SELECTION:

The study was approved by the Institutional Ethical committee. About 80 patients in the age group of 35- 60 years who attended the out patient department , Department of Periodontics, Tamilnadu Government Dental College,Chennai between June and October 2010 participated in the study . the patients were divided into 2 groups, 40 subjects with generalized chronic periodontitis and 40 healthy subjects taken as control.

A written informed consent was obtained from all patients. A complete history and clinical features of the subjects attending study were obtained. Prior to blood sample collection , plaque index, bleeding index, probing depth, clinical attachment loss were recored.

About 10ml of venous blood samples were collected from the patients with or without periodontitis for ELISA and spreading analysis test.

INCLUSION CRITERIA:

1. Patients willing for voluntary participation and signing the informed consent.
2. Age : 35-60 years
3. Gender : Both males and females
4. Patients with generalized chronic periodontitis with minimum 20 teeth.

EXCLUSION CRITERIA:

1. Trauma or tooth extraction within 2 weeks
2. Patients drug history(antibiotics, anticoagulants) for past 2 months.
3. Any chronic infectious diseases.

4. Pregnancy
5. Anemia, bleeding disorders or any other hematological disorder
6. Cardiac diseases
7. Stroke
8. Habits like smoking , pan chewing, alcohol.

The selected patients were divided into two groups (Control and Study groups) based on the following criteria:

Control Group:

This included 40 healthy subjects who exhibited the following features:

- No Bleeding on Probing
- Absence of Clinical Attachment loss as determined by CAL (Clinical Attachment Level) measurements i.e. CAL=0.
- Good oral hygienic status

Study Group:

The diagnosis of Chronic Periodontitis was established on the basis of Classification of Periodontal Diseases, AAP 1999.

- Bleeding on Probing
- Probing pocket depth in mm
- Presence of signs of Clinical Attachment Loss as determined by CAL measurements $\geq$ 5mm.
- At least a minimum 20 teeth were should be present.
- Poor oral hygiene

STUDY PROTOCOL:

1 Institutional ethical committee approval
2. Medical History and Informed Consent
3. Periodontal Examination using clinical parameters namely, Bleeding Index, Plaque Index, Probing Pocket Depth and Clinical Attachment Level.
4. Collection of blood samples
5. Estimation of serum soluble P- selectin level by ELISA method.
6. Evaluate the Platelet morphology and grading the platelet aggregation.

Following selection of subjects, written informed consent was obtained from all the subjects selected for the study after explaining the study procedure. Examination was preceded by a thorough medical and dental history of the subjects. Intra-oral examination was done using mouth mirror and William's Periodontal Probe. Periodontal Evaluation was done by measuring the Bleeding Index, Plaque Index, Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL)

CLINICAL PARAMETERS

GINGIVAL BLEEDING INDEX (Ainamo & Bay 1975)[1]

Teeth examined - All teeth

Surfaces examined - 6 sites for each tooth (Mesiobuccal,Midbuccal, Distobuccal, Mesiolingual, Midlingual and Distolingual)

The presence or absence of bleeding is determined by gentle probing of the gingival crevice with a periodontal probe.

Criteria for Scoring

Positive score (+) - Presence of bleeding within 10 seconds

Negative score (-) - Absence of bleeding

$$\% \text{ of bleeding sites} = \frac{\text{Total number of positive score}}{\text{Total number of surfaces of all teeth}} \times 100$$

PLAQUE INDEX (Silness and Loe 1964)[23]

Teeth examined – All teeth

Surfaces examined – 4 sites for each tooth (disto-facial, Facial, Mesio-facial, lingual/palatal)

Criteria for Scoring:

Score 0	No plaque
Score 1	Plaque not visible to the naked eye, detected by explorer
Score 2	Thin to moderate accumulation of soft deposits within the gingival pocket or on tooth, visible to the naked eye
Score 3	Abundance of soft matter within gingival pocket or on tooth surface and margin, inter-dental area stuffed with soft debris

Calculation : Plaque index for a tooth= total score from 4 areas/4

Plaque index for the individual = Total Plaque indices for all teeth / No. of teeth examined .

Interpretation: Score 0 – Excellent oral hygiene

0.1 to 0.9 – Good oral hygiene

1.0 to 1.9 – Fair oral hygiene

2.0 to 3.0 - Poor oral hygiene

PROBING POCKET DEPTH (PPD) (Grant 1965)[41]

Probing Pocket Depth was measured from the gingival margin to the base of the pocket using William's Periodontal Probe. The probe was passed within the gingival sulcus along the circumference of the tooth.

Three measurements were made on the buccal aspect and three on the lingual aspect of each tooth – total of six sites per tooth (Mesiobuccal, Midbuccal, Distobuccal, Mesiolingual, Midlingual, Distolingual).

CLINICAL ATTACHMENT LEVEL (CAL) (Carranza)[23]

Clinical Attachment Level was measured from the Cemento – Enamel Junction (CEJ) to the base of the pocket using William's Periodontal Probe.

- When the gingival margin was located on the anatomic crown, the level of the attachment was determined by subtracting from the probing pocket depth, the distance from the gingival margin to the CEJ. If both were the same, the loss of attachment was calculated to be zero.
- When the gingival margin coincided with the CEJ, the loss of attachment was calculated as equaling the probing pocket depth.
- When the gingival margin was located apical to the CEJ, the loss of attachment was greater than the probing pocket depth and therefore the distance between the CEJ and the gingival margin were added to the PPD.

Three measurements were made on the buccal aspect and three on the lingual aspect of each tooth – total of six sites per tooth (Mesiobuccal, Midbuccal, Distobuccal, Mesiolingual, Midlingual, and Distolingual).

Armamentarium:

Mouth mirror

William's periodontal probe

Head cap

Gloves and mask.

BLOOD SAMPLE COLLECTION AND STORAGE:

Fasting venous blood samples about 10ml was collected without stasis using 20G needle by venipuncture in the antecubital fossa after skin preparation. About 5ml of blood is allowed to stand in plain vacutainer tube for 30 min to separate the serum and centrifuged at 3000 rpm for 10 min. Then 500µl of serum sample was divided in aliquots and stored at -20°C until analysis.

Remaining 5 ml of blood is divided into 2 parts. About 4ml of blood immediately transferred into 3.8% sodium citrate solution containing vacutainer tubes for PRP preparation, and about 1ml of blood transferred into EDTA containing vacutainer tubes and used for whole blood analysis.

PROCEDURE:

I. ELISA METHOD:

A soluble form of P-selectin, which might represent a proteolytic fragment or a soluble splice variant lacking the trans membrane domain, is found in serum which is estimated by **Diaclone ELISA Kit, France.**

Contents of test kit:

1. **96-wells microtiter plates**: Ready-to-use

2. **Standard**: 140 ng/ml, 2 vials ,

   Reconstituted with the volume of standard diluent indicated on the vial.

3. **Standard Diluent Buffer**: 1 vial

   25 ml dilute 10 times in distilled water

4. **Biotinylated anti-CD62P** : 1 vial ,Dilute in biotinylated antibody diluents

5. **Biotinylated Antibody Diluent**: 1 vial (7ml)

   Ready-to-use

6. **Streptavidin- HRP** : 2 vials

   0.5ml of HRP-Diluent before further dilutions

7. **HRP Diluent** : 1 vial (23 ml)

   Ready-to-use

8. **Washing Buffer** : 1 vial (10 ml) 200X concentrate.

   Dilute in distilled Water

9. **Chromogen TMB** : 1 vial (11 ml) ; Ready-to-use

10. **H_2SO_4:** Stop Reagent ,1 vial

    Ready-to-use

Armamentarium:

Test tubes

Eppandorf tubes

Plastic rack

Measuring jar

Bowls

Autoclaved plastic tips

Micropipette

ELISA washer

ELISA reader

ASSAY METHOD

i. Before use, all reagents are mixed thoroughly without making foam.

ii. Number of micro wells strips required to test the desired number of samples, plus appropriate number of wells needed for running blanks and standards are determined. Each sample, standard and blank should be assayed in duplicate .

iii. About 100 µl of standard diluents is added to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2. Reconstitute the standard vial with the appropriate volume .Pipet 200 µl of standard into wells A1 and A2 .About 100 µl transferred from A1 and A2 to B1 and B2 wells. Contents are mixed by repeated aspirations and ejections. Taking care not to scratch the inner surface of microwells. The procedure is repeated from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and to create two parallel rows of CD62P standard dilutions ranging from 140 to 4.4 ng/ml. 100 µl from the content of the last microwells used (F1, F2) was discarded.

iv. Alternatively these dilutions can be done in separate tube and the diluted standard pipetted directly into wells.

v. About 100 µl of standard diluent is added to the blank wells (G1-G2).

vi. About 100 µl of serum sample was added to sample wells.

vii. The plate is covered and incubated for 1 hour at temperature (18°C - 25°C).

viii. T he cover is removed and washed in the plate following manner:

a. The liquid from each well is aspirated

b. About 0.3 ml of washing solution is added into each well ;

c. The content of each well was aspirated again

d. steps a) and b) are repeated two times.

ix. Biotinylated anti-CD62P solution prepared.

x. About 50 µl of diluted biotinylated anti-CD62P was added to all wells.

xi. Covered and incubated for 1 hour at room temperature.

xii. Washed as described at step viii

xiii. HRP solution prepared just before to use . 100 µl of HRP solution is dispensed into all wells, including the blank wells. The cover was put back

xiv. The microwell strips were incubated at room temperature for 30 minutes.

xv. The plate cover was removed and wells emptied .The microwell strips washed as per step viii

xvi. About 100 µl of ready-to-use TMB substrate solutionwas added into all wells, including the blank wells and incubated in the dark for 5-15 min minutes at room temperature. Avoiding direct exposure to light by wrapping the plate in aluminium foil. Incubation time of the substrate solution is usually determined by the ELISA reader performances: many ELISA readers record absorbance only up to 2.0 O.D. The O.D. values of the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly readable.

xvii. The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of H_2SO_4: stop reagent into each well, including the blank wells, to completely and uniformly inactivate the enzyme. Results were read immediately after the addition of H_2SO_4: stop reagent.

xviii. The absorbance of each well was read on a spectrophotometer using 450 nm wavelength. .

ASSAY PROCEDURE SUMMARY

About 100µl of sample added.

⇓

Incubated for 1 hour at room temperature

⇓

Washed three times

⇓

About 50 µl of biotinylated detection antibody added

⇓

Incubated for 1 hour at room temperature

⇓

Washed three times

⇓

About 100 µl of Streptavidin-HRP added

⇓

Incubated for 30 min at room temperature

⇓

Washed three times

⇓

About 100 µl of ready-to-use TMB added
(Protect from light. Let the color develop for 5-15 min)

⇓

Added 100 H_2SO_4 (stop reagent)

⇓

Read Absorbance at 450 nm

Calculation of results: The optical density of each sample was plotted against its concentration and a curve was drawn through the points

II. SPREADING ANALYSIS TEST:

This test was adapted from the method introduced by ***Marx 1960*** and modified by ***Breddin 1965.***[14]

Preparation of citrated platelet rich plasma (cPRP)

About 4ml was blood drawn and transferred into vacutainer tube mixed with sodium citrate in 1: 9 ratio. Then sample was centrifuged at 2000 rpm for 15min without break at room temperature. The resulted cPRP was transferred carefully by micro-pipette (using new disposable plastic tips) into a plastic collection tube to get ready for further analysis. The aspiration of the cPRP was performed with a specific care not to disturb the lower layers of leukocytes and erythrocytes. The cPRP was pipetted gently on the inner wall of the collection tube.

REAGENTS USED :

1.Sodium citrate solution : 0.3% solution used for lowering the viscosity of the blood reactions.

2. Formaldehyde solution 11% : One liter (i.e., 1.04 kg) stock solution for analysis stabilized with 10% methanol (toxic);

3. Potassium permanganate solution 0.1% N: 3.161 g $KMnO_4$/ 100 mL; and stored in dark place.

4. Giemsa stain: Azur-Eosin-Methylene blue solution for microscopy ,and stored at15-25 °C.

Armamentarium:

Plastic slides (75mmx 25mm)

Micropipette

Plastic tips

Light microscope x100

PROCEDURE:

Platelet counts was assessed with a ***Sysmex Automated Haematology Analyzer KX-21*** and each PRP samples was adjusted to 0.3×10^8/ml with 0.3% sodium citrate.

1. About 2-4 mL of the resultant platelet suspension is poured on a specific athrombogenic plastic slide and incubated at room temperature for 30 min allowing the platelets to adhere and spread on the slide.

2. The slide was rinsed carefully with the isotonic Na-citrate solution

3. Fixation step: The slide was allowed to stand in 10% formaldehyde solution for 7 mins.

4. The slide is rinsed with isotonic Na-citrate solution.

5. Oxidation step: The slide was allowed to stand in the 0.1N potassium permanganate solution for 5 mins.

6. The slide was rinsed with isotonic Na-citrate solution.

7. Staining is accomplished by using Giemsa's solution for 60 mins.

8. The slide was rinsed gently with distilled water and air dried.

9. The Slide was examined by light microscopy at the magnification of x100

Platelet size and morphology:

The preparations of Platelet spreading were examined by light microscopy. According to ***Scharrer 1985*** [97]**,** five types of PLT can be distinguished in healthy individuals:

(1) Giant forms with a surface of more than 200 µm.

(2) Big forms with minimum one diameter more than 10 µm.

(3) Small forms with an average diameter less than 10 µm.

(4) Intermediate forms (round, etc.,) which are not completely spread and have little hyalomere between pseudopodia.

(5) Spider forms which are not spread Platelet with one or more short and relative thick pseudopodia.

The forms 1-3 are defined as spread Platelet forms while the forms 4 and 5 are not spread. In this study , we examined only big , small and spider forms for percentage calculation.

During microscopic examination, hundred Platelet were counted in each Platelet spreading preparation. The findings were recorded on an application that was designed to include the complete descriptions of the examined preparations as follows:

A.) The technical quality of the preparation (thickness, coloring, dirts if any, etc.) was differentiated. The thickness of the preparations reflect firstly the Platelet count of the tested sample and secondly the adhesion of Platelet.

B.) The relative presence in percentages (%) of the spread (big, and small) and non-spread (spiders).

C.) The grades of Platelet aggregates (Aggr) were assessed semi quantitatively, and differentiated according to grading system given by ***Breddin HK, Bauke J 1965.***[14]

Platelets aggregation grading:

In this test, ***reversible platelet aggregation*** means the sticking of platelets to each other without fusion, with their membranes intact. In ***irreversible aggregation***, platelets are fused and membranes are partly destroyed. The platelet aggregation is graded according to the following characteristics:

- **Grade 1:** Platelets are seen single with only a few reversible aggregates and no irreversible aggregates.
- **Grade 2:** More reversible aggregates are found, but there are no or only very few irreversible aggregates; there is no obvious reduction in single platelets.
- **Grade 3:** An increase in reversible aggregates is evident, often with irreversible aggregation at the centre; the number of platelet is clearly diminished.
- **Grade 4:** There are more irreversible than reversible aggregates; single platelets are rare.
- **Grade 5:** There is complete irreversible aggregation; single platelets are practically missing.

The grades 1 and 2 after rotation were taken as normal, and the grades 3, 4, and 5 as pathological aggregability.

WHOLE BLOOD ANALYSIS: Citrated blood was used for whole blood analysis by using ***Sysmex Automated Haematology Analyzer .*** Total WBC, platelets, neutrophils, lymphocytes, RBCs cell were estimated in both control and Periodontitis subjects.

Photograph No: 1 Healthy control group

Photograph No: 2 Study Group - Moderate Chronic Periodontitis

Photograph No: 3 Study group - Severe chronic periodontitis

Photograph No. 4 Pocket depth >5mm in severe periodontitis

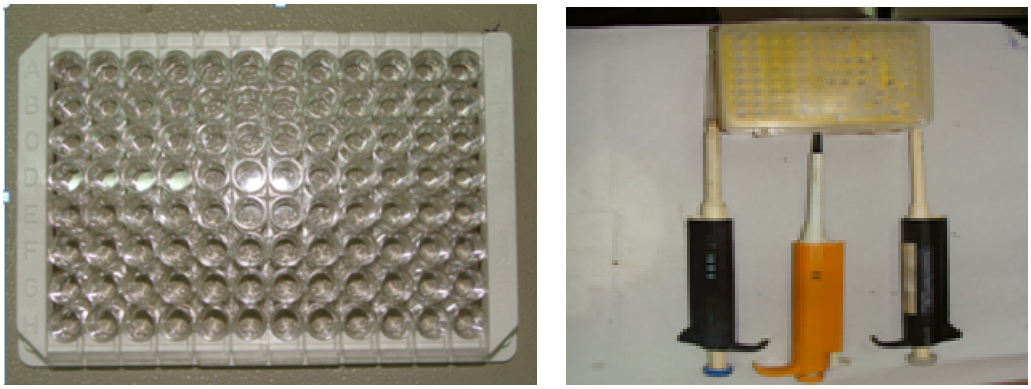
Photograph No. 5 Armamentarium for clinical examination

Photograph No. 6 Collection of blood sample

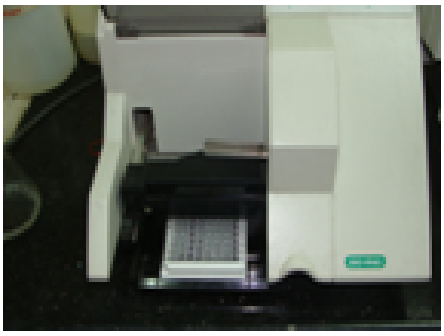
Photograph No.7 Serum samples & Armamentarium for sample transportation

Photograph No.8 Deep freezer and samples stored at -20 ^{0}C

Photograph No.9 ELISA KIT & its contents



Photograph No.10 96well microplate of ELISA KIT and micropipettes &tips



Photograph No.11 ELISA washer and Spectrophotometer

Photograph No.12 Light microscope and Sysmex Haematology analyser

STATISTICAL ANALYSIS

The statistical analysis was done using the computer software program SPSS version 12.

Mean and Standard Deviation were estimated for different variables in each study group.

Mean values were compared between two study groups by using either ***Student's Independent t-test*** or ***Mann-Whitney U-Test***.

Pearson's chi-square test was done to compare the proportions in two study groups.

In the present study, ***P-value <0.05*** was considered as the level of significance.

Statistical Formulae Used For Data Analysis

Pearson's Chi-square Test

The formula used was

$$\chi^2 = \sum_{i=1}^{n} \left\{ \frac{(O-E)^2}{E} \right\}$$

Where O = Observed frequency in a cell

E = Expected frequency in a cell

Mann-Whitney U-Test

The formula is

$$z = \frac{\left[T - \frac{n1(n1+n2+1)}{2}\right]}{\sqrt{\frac{n1n2(n1+n2+1)}{12}}}$$

Where n1 and n2 are the sample sizes in Group I and Group II respectively.

T is the sum of the ranks for the n1 observations.

Student's Independent t-test

The formula used is

$$t = (\overline{X_1} - \overline{X_2}) \Big/ \sqrt{S_p^2/n_1 - S_p^2/n_2}$$

where $S_p^2 = [(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2] \Big/ (n_1 + n_2 - 2)$

$\overline{X_1}$ and $\overline{X_2}$ are the sample means;

RESULTS

Eighty subjects were included in the present study. The subjects were categorized into two groups – cases (n = 40) and controls (n = 40).

Table I& II shows the master chart of study and control group with the variables, clinical parameters and tests done in both groups in the study.

Table III shows the comparison of age between study and control groups. There is no statistical difference in the distribution of sex in both the groups.

Table IV shows the comparison of gender distribution between study and control group. There is no statistical difference in the distribution of gender in both groups.

Table V shows the comparison of mean PPD & CAL(mm) of control and study group. The mean PPD value of control group is 2.11mm and of study group is about 4.9mm and the mean CAL value of cases 5.8mm.

Table VI & Figure I shows mean number of total WBC, RBC,& Hb % for control and study group. WBC counts in study group($8.04\pm 1.84 \times 10^3$)in contrast to the control group ($5.46\pm1.84 \times 1.23 \times 10^3$) with statiscal significance.($P < 0.001$).

Mean value of RBC count (control $3.99 \pm 0.58 \times 10^6$ study group $4.41 \pm 0.51 \times 10^6$) with P-value 0.001and Hb% (control 11.43 ± 1.32 gm % study group 12.20 ± 1.87 gm% with P-value 0.035) .Statiscally the mean value of RBC count, Hb % among control and study group is significant.

Table VII& Figure II and III shows that the mean Platelets, Neutrophils, lymphocytes count. In study group mean platelet count was found to be 281.25 ± 84.5 and the control group is about 200.98 ± 56.9 which found to be statistically highly significant (P=<0.001).

Mean value of neutrophil count in control group 2.94 ± 0.68 x 10^3& study group 4.09 ± 1.50 x 10^3) and lymphocyte count in control 1.95 ± 0.55 x 10^3 & study group 2.83 ± 0.72 x 10^3) . It was found to be statiscally highly significant (P<0.001) among the study group

Table VIII & Figure IV shows mean P-selectin expression in both control and study group. Statistical analysis shows that the mean value for control group is 4.97 ± 16.56 ng / mL and study group 13.05 ± 29.94 ng / mL which was significantly higher than control group with P-value 0.001.

Table IX & Figure V shows Pearson's correlation of P- selectin expression and mean CAL level of controls and cases. Result shows that increased P-selectin expression is positively associated with CAL level .(P- value < 0.001).

Table X and Figure VI &VII shows platelet morphological changes in control and study group. Small form – mean value for control group is 75.83% ± 14.24% while for study group is 39.08 %. ± 21.59 ;Big form – mean value for control group 0.80% ± .35% while for study group 0.48% ± 1.3%and Spider form- mean value for control group 23.88% ±14.13 while study group 59.32% ±. 23.42 .The observation showed high statistical significance with P- value <0.001 for small and spider form and no statistical significance for big form P =0.075.

Table XI & Figure VIII shows distribution of platelet aggregation grading for control and study group . **Grade I** in control group shows 77.8% and study group shows 22.2%.**Grade II** – control group 75.5% and study group 24.3% ; **Grade III** -

control group 15.6% and study group 84.4%; **Grade IV** – control group 0% and 100% for study group.

Within the group correlation showed grade II (70%) found maximum among the control group followed by grade I (17.5%) Grade III (12.5%) and grade IV (0%) . For study group grade III (67.5%) was found to be maximum followed by grade II (22.3%) and grade I & grade IV (5.0% each) with P-value <0.001.

Table XII & Figure IX shows that correlation between P-selectin expression and platelet aggregation grading. Pearson's correlation shows a positive correlation between these two variables with the P-value 0.005 among the study and control group.

TABLE -I MASTER CHART-CONTROL

								Platelet Morphology				Whole Blood Analysis					
S.No	C.No	Age	Sex	PPD mm	CAL mm	% OF Sites Bleeding on Probing	P-Selectin ng/mL	Small	Large	Spider	P.Agg Grading	WBC 10^3	RBC 10^6	Hb gm	Platelet 10^3	Lymphocyte 10^3	Neutrophil 10^3
1	C2	45	F	2.05	0	9.6	0	56	-	44	++	5.8	4.07	11.5	234	2.3	2.7
2	C3	38	F	2.18	0	3.22	1	83	-	17	++	3.3	4.03	11.2	138	1.3	2.5
3	C6	58	M	2.07	0	5	0	73	2	15	+	6.2	3.47	9.7	178	1.6	4.1
4	C7	36	M	2.11	0	6.6	0	44	1	55	++	4.5	3.85	10.6	126	1.3	2.3
5	C8	35	M	2.12	0	4.65	0	80	-	20	++	7.3	4.12	12	198	2.4	4.4
6	C9	35	M	2.06	0	5	0	55	-	35	++	6.6	3.98	11.4	221	2.5	3.5
7	C10	58	F	2.15	0	4.9	0	45	-	55	++	4	3.62	10.5	161	1.4	2.3
8	C11	42	M	2.2	0	5.37	0	69	-	31	++	4.3	4.02	10.4	134	1.5	2.3
9	C13	45	F	2.04	0	5	0	85	-	15	+	5.8	4.05	11.4	158	1.6	3.7
10	C14	32	F	2.03	0	4.68	0	73	-	37	++	6.9	4.34	11.9	248	2.6	3.8
11	C15	62	M	2.1	0	4.16	0	62	-	38	++	5.7	4.03	11.5	239	1.5	3.6
12	C16	38	F	2.07	0	3.22	0	61	-	39	+	7.4	4.03	11	213	2.4	4.3
13	C17	50	F	2.13	0	9.9	1	85	-	15	++	6.5	3.93	12.5	197	2.3	4
14	C18	40	F	2.08	0	6.17	0	71	-	29	++	5.3	4.44	14	175	2.3	2.3
15	C19	38	F	2.05	0	4.28	0	96	-	4	++	6.9	4.44	13.3	374	3.2	3
16	C21	40	M	2.16	0	4.14	0	55	-	45	+++	5.8	4.21	12.8	237	1.8	3.5
17	C22	39	F	1.44	0	6.66	1	76	-	24	++	6.3	3.09	11.9	203	2.8	2.4
18	C23	38	F	2.36	0	9.97	61	92	-	8	+++	6.9	3.61	10.2	230	2	2.2
19	C24	46	F	2.04	0	4.9	0	90	-	10	+++	4.9	3.35	8.3	163	2	2.1
20	C25	35	M	2.06	0	6.07	1	81	-	19	++	6.7	5.24	13.5	203	2.3	3.1

								Platelet Morphology				Whole Blood Analysis					
S.No	C.No	Age	Sex	PPD mm	CAL mm	% OF Sites Bleeding on Probing	P-Selectin ng/mL	Small	Large	Spider	P.Agg Grading	WBC 10_3	RBC 10_6	Hb gm	Platelet 10_3	Lymphocyte 10_3	Neutrophil 10_3
21	C27	39	F	2.06	0	5.13	0	80	-	20	++	6.6	4.34	12.7	249	2.6	3.4
22	C28	40	F	2.05	0	3.12	0	58	-	43	++	5.9	3.86	10.2	372	1.8	3.3
23	C29	54	M	2.12	0	4.76	1	90	-	10	++	8.8	5.4	15.4	242	3.2	4.3
24	C31	48	F	2.06	0	5.37	0	82	-	18	++	4.2	3.25	10	210	0.8	2.7
25	C33	48	F	2.13	0	4.9	2	81	-	19	+++	3.7	3.15	11.3	233	1.2	2.2
26	C34	37	F	2.09	0	5	0	85	-	15	++	3.9	4.44	10.6	86	1.6	2.2
27	C36	41	F	2.01	0	3.22	0	71	-	29	++	4.9	3.76	10.5	100	1.9	2.6
28	C38	38	M	2.2	0	6.6	0	96	-	4	++	5	3.7	10.3	155	1.8	2.3
29	C39	39	F	2.12	0	4.28	0	100	-	0	++	5.9	3.94	12.5	160	2.5	2.8
30	C40	36	M	2.12	0	5	0	94	-	6	+	4.8	4.24	11.1	179	1.6	3
31	C41	40	M	2.06	0	4.17	0	73	-	27	++	4.6	3.38	12.8	198	1.5	2.8
32	C42	36	F	2.02	0	6.18	1	68	-	32	++	3.7	3.79	11.5	178	1.3	2.3
33	C43	44	M	2.18	0	4.46	2	83	-	17	+	4.1	4.43	12.6	192	1.9	2.6
34	C44	38	M	2.08	0	6.68	1	92	-	8	++	5	3.28	10.2	202	1.7	2.5
35	C45	39	M	2.13	0	6.1	0	87	-	13	++	6.2	5.36	10.8	216	1.4	3.1
36	C46	40	M	2.11	0	7.4	0	78	-	22	+	4.3	3.17	11.1	161	2.5	2.7
37	C47	37	F	2.32	0	8.02	61	55	-	45	+++	4.8	3.78	11.6	213	2.3	3.2
38	C48	42	F	2.13	0	6.42	1	81	-	19	+	5.2	3.96	10.2	265	2.1	2.1
39	C49	49	F	2.17	0	5.12	0	78	-	22	++	4.1	3.45	10.5	195	1.7	2.6
40	C51	54	M	2.98	0	7.58	65	69	-	31	++	5.6	5.1	11.5	203	1.4	2.8

TABLE -II MASTER CHART-CASES

							Platelet Morphology					Whole Blood Analysis					
S.No	C.No	Age	Sex	PPD mm	CAL mm	% OF Sites Bleeding on Probing	P-Selectin ng/mL	Small	Large	Spider	P.Agg Grading	WBC 10^3	RBC 10^6	Hb gm	Platelet 10^3	Lymphocyte 10^3	Neutrophil 10^3
41	P1	48	M	4.46	5.54	84	1	43	3	54	+++	5.2	3.34	9.1	179	1.9	2.3
42	P2	32	F	6.22	6.98	100	76	55	6	38	+++	11.2	4.18	12.7	278	2.6	2.3
43	P8	40	M	6.02	6.72	83.68	2	55		45	+++	5.3	3.92	9.7	127	3.1	2.4
44	P9	52	M	6.45	7.59	100	90	31	1	68	+++	7.7	5.04	14.3	292	3	4
45	P10	30	F	5.92	6.53	84.47	2	5	-	95	++	6	3.72	9.3	227	2.4	2.6
46	P11	37	F	4.41	5.12	51.13	0	5	1	94	+	6.8	3.86	10.2	210	2.3	2.5
47	P12	40	M	6.8	7.4	57.03	2	32	-	68	++	5.5	4.46	13.3	279	1.5	3.6
48	P13	42	F	5.07	5.4	67.08	1	6	-	94	++	7.9	4.98	14.3	247	2.9	3.9
49	P14	45	F	4.37	5.18	43.04	0	85	-	15	+++	5	4.14	10.9	266	2.2	2.2
50	P15	32	M	3.39	4.48	55.17	0	53	3	44	+++	7.9	4.5	13.3	174	2.1	4.3
51	P16	40	F	5.09	5.68	93.18	1	39	-	68	+++	11.8	4.98	13.2	281	3.5	7.2
52	P17	48	M	6.18	7.32	100	80	22	-	78	++	9.4	4.65	11.2	472	3.9	5
53	P18	30	M	3.98	4.44	95.17	1	48	-	52	+++	8.6	5.23	14.7	256	2.8	4.2
54	P19	49	M	5.65	6.49	91.18	2	32	-	6.8	++	6.9	4.97	16.8	242	2.3	3.1
55	P20	38	M	4.48	5.02	83.14	1	50	-	50	+++	8.4	4.37	11.7	340	1.8	5.8
56	P21	31	F	6.38	7.32	94.4	2	47	-	53	+++	6.7	4.84	14.2	237	2.5	3.4
57	P22	38	M	4.12	5.09	67.16	0	17	-	83	+++	7.8	4.83	13.8	230	2.4	3.4
58	P23	60	M	3.88	4.85	48.44	0	48	-	52	+++	8.3	3.45	12.7	398	2.7	2.3
59	P24	48	M	4.63	4.95	73.17	1	7	-	93	+++	6.8	4.32	12.3	365	2.6	2.4
60	P25	38	F	4.78	5.68	74.38	1	45	5	50	+++	7.8	4.35	11.7	317	3.3	3.2

								Platelet Morphology				Whole Blood Analysis					
S.No	C.No	Age	Sex	PPD mm	CAL mm	% OF Sites Bleeding on Probing	P-Selectin ng/mL	Small	Large	Spider	P.Agg Grading	WBC 10_3	RBC 10_6	Hb gm	Platelet 10_3	Lymphocyte 10_3	Neutrophil 10_3
61	P26	43	F	6.4	7.2	87.18	3	11	-	89	++	8.8	5.02	12.9	259	4.1	4.5
62	P27	43	F	4.47	5.02	64.04	1	19	-	81	+	8.1	5.2	13.6	275	4.5	4.2
63	P28	32	F	6.12	7.02	81.08	2	44	-	66	+++	7.6	4.07	10.9	423	4.3	3.9
64	P29	40	F	4.17	5.02	57.13	0	20	-	80	+++	8.9	4.18	11.3	402	4.5	4
65	P30	42	M	6.29	7.92	100	114	80	-	20	++++	7.3	4.9	13.9	224	2.3	2.6
66	P31	60	M	6.03	6.18	73.15	1	65	-	35	+++	5.2	3.42	9.3	96	2.1	2.8
67	P32	52	F	6.38	7.59	98.12	65	72	-	28	+++	9.2	4.27	11.4	390	3.2	4.6
68	P33	38	F	4.32	4.98	73.02	0	21	-	79	++	6.3	3.95	11.6	172	2.4	3.3
69	P34	39	M	5.08	5.71	81.2	1	22	-	78	+++	6.89	3.98	11.6	163	2.6	3.4
70	P35	40	F	6.05	6.73	89.02	2	54	-	46	+++	7.3	4.59	8.7	364	3.1	3.5
71	P36	32	M	4.18	4.93	91.04	1	29	-	71	+++	7.5	4.63	8.6	357	3.2	3.6
72	P37	47	F	3.38	4.16	45.16	0	25	-	75	+++	8.8	4.94	14.6	240	2.7	5
73	P38	46	F	3.96	5.02.	45.47	0	62	-	38	+++	9.3	4.92	14.8	249	2.7	5.4
74	P39	54	F	4.67	5.35	57.08	1	80	-	20	+++	9.3	4.33	12.9	264	2.2	6.2
75	P40	49	M	6.2	6.45	87.19	65	35	-	65	+++	9	4.15	11.8	307	2	6.1
76	P41	50	F	3.45	4.94	100	0	44	-	56	+++	12.4	4.88	12.9	411	3.2	7.4
77	P42	57	F	6.04	6.93	75.08	3	56	-	44	+++	12.6	4.84	13	383	3.2	7.7
78	P43	51	F	3.18	3.98	51.43	0	55	-	45	++	8.9	3.94	11.4	300	2.7	5.8
79	P44	50	M	3.17	4.43	57.05	0	16	-	84	+++	9.1	3.88	11.3	293	2.7	5.9
80	P45	45	M	4.02	4.98	65.18	0	28	-	72	++	8	4.29	12.2	261	3.5	3.7

TABLE –III : COMPARISON OF AGE BETWEEN CONTROL & STUDY GROUP

	Group	**N**	**Mean**	**Std. Deviation**	**P-value**
Age in years	Control	40	42.23	7.123	**0.589 (NS)**
	Study	40	43.20	8.106	

TABLE –IV COMPARISON OF GENDER BETWEEN CONTROL & STUDY GROUP

			Group		**Total**	**P- value**
			Control	**Study**		
Sex	**Male**	Count	17	19	36	**0.653 (NS)**
		% within Sex	47.2%	52.8%	100.0 %	
		% within Group	42.5%	47.5%	45.0%	
	Female	Count	23	21	44	
		% within Sex	52.3%	47.7%	100.0 %	
		% within Group	57.5%	52.5%	55.0%	
Total		Count	40	40	80	
		% within Sex	50.0%	50.0%	100.0 %	
		% within Group	100.0%	100.0%	100.0 %	

TABLE V: MEAN PROBING DEPTH AND CAL OF CONTROL & STUDY GROUP

	Group	N	Mean	Std. Deviation	P-value
PPD in mm	Control	40	2.1160	.19013	**0.000**
	Study	40	4.9960	1.09803	**0.000**
CAL in mm	Control	40	.0000	.00000	**0.000**
	Study	40	5.8080	1.10563	**0.000**

TABLE-VI : COMPARISON OF WBC,RBC,& Hb. BETWEEN CONTROL & STUDY GROUP

	Group	N	Mean	Std. Deviation	P-Value
WBC	Control	40	5.460	1.2345	**<0.001****
	Study	40	8.037	1.8352	**(SIG)**
RBC	Control	40	3.9925	.57687	**<0.001****
	Study	40	4.4128	.50902	**(SIG)**
HB	Control	40	11.425	1.3210	**0.035***
	Study	40	12.202	1.8725	

TABLE –VII: COMPARISON OF PLATELETS, LYMPHOCYTES,NEUTROPHIL COUNTS BETWEEN CONTROL & STUDY GROUP

	Group	**N**	**Mean**	**Std. Deviation**	**P-Value**
No. of Platelet	Control	40	200.98	56.914	**<0.001**
	Study	40	281.25	84.543	
Lymphocyte	Control	40	1.948	.5547	**<0.001**
	Study	40	2.825	.7221	
Neutrophil	Control	40	2.940	.6778	**<0.001**
	Study	40	4.093	1.4984	

TABLE -VIII

COMPARISON OF P- SELECTIN IN CONTROL AND STUDY GROUP

	Group	**N**	**Mean**	**Std. Deviation**	**P- value**
P-Selectin	Control	40	4.97	16.558	**0.001****
	Study	40	13.05	29.943	**(Sig)**

TABLE IX : COMPARISON OF P-SELECTIN EXPRESSION TO SEVERITY OF CAL IN STUDY GROUP

		P-Selectin
CAL in mm control	Pearson Correlation	**.(a)**
	Sig. (2-tailed)	**.**
	N	**40**
CAL in mm Study	Pearson Correlation	**.608(**)**
	Sig. (2-tailed)	**.000**
	N	**40**

**** Correlation is significant at the 0.01 level (2-tailed).**

TABLE – X: COMPARISON OF PLATELET MORPHOLOGY OF CONTROL & STUDY GROUP

	Group	**N**	**Mean**	**Std. Deviation**	**P- value**
Small	Control	40	75.83	14.240	**<0.001****
	Study	40	39.08	21.590	**(Sig)**
Large	Control	40	.08	.350	<0.075
	Study	40	.48	1.358	**(NS)**
Spider	Control	40	23.88	14.137	**<0.001****
	Study	40	59.32	23.428	**(Sig)**

TABLE- XI: PLATELET AGGREGATION GRADING

			Group		Total	P-Value
			Control	Periodontitis		
Platelet Agg. Grading	+	**Count**	7	2	9	**<0.001** (SIG)**
		% within Platelet Aggregation Grading	77.8%	22.2%	100.0 %	
		% within Group	17.5%	5.0%	11.3%	
	++	**Count**	28	9	37	
		% within Platelet Aggregation Grading	75.7%	24.3%	100.0 %	
		% within Group	70.0%	22.5%	46.3%	
	+++	**Count**	5	27	32	
		% within Platelet Aggregation Grading	15.6%	84.4%	100.0 %	
		% within Group	12.5%	67.5%	40.0%	
	++++	**Count**	0	2	2	
		% within Platelet Aggregation Grading	.0%	100.0%	100.0 %	
		% within Group	.0%	5.0%	2.5%	
Total		**Count**	40	40	80	
		% within Platelet Aggregation Grading	50.0%	50.0%	100.0 %	
		% within Group	100.0%	100.0%	100.0 %	

TABLE XII: CORRELATION OF sP- SELECTIN LEVEL AND PLATELET AGGREGATION GRADING

		P-Selectin	**Platelet Aggregation Grading**
P-Selectin	Pearson Correlation	1	**.309(**)**
	Sig. (2-tailed)	.	**.005(Sig)**
	N	80	**80**
Platelet Aggregation Grading	Pearson Correlation	**.309(**)**	1
	Sig. (2-tailed)	**.005 (Sig)**	.
	N	**80**	80

FIGURE I: COMPARISON OF WBC ,RBC & Hb% IN CONTROL & STUDY GROUP

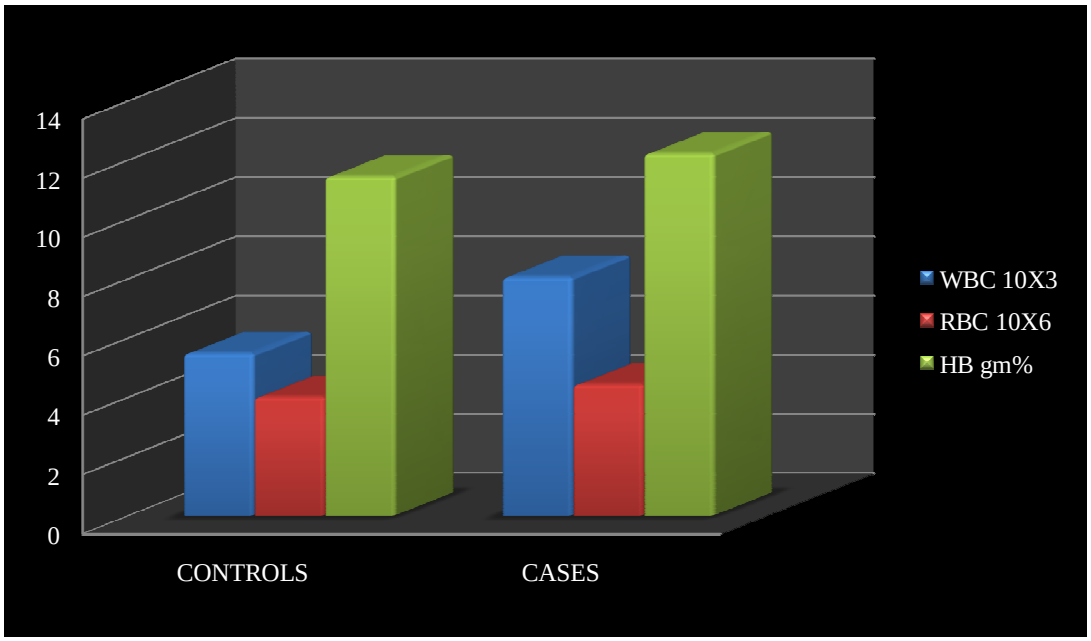


FIGURE II : COMPARISON OF LYMPHOCYTES, NEUTROPHIL COUNTS IN CONTROL &STUDY GROUP .

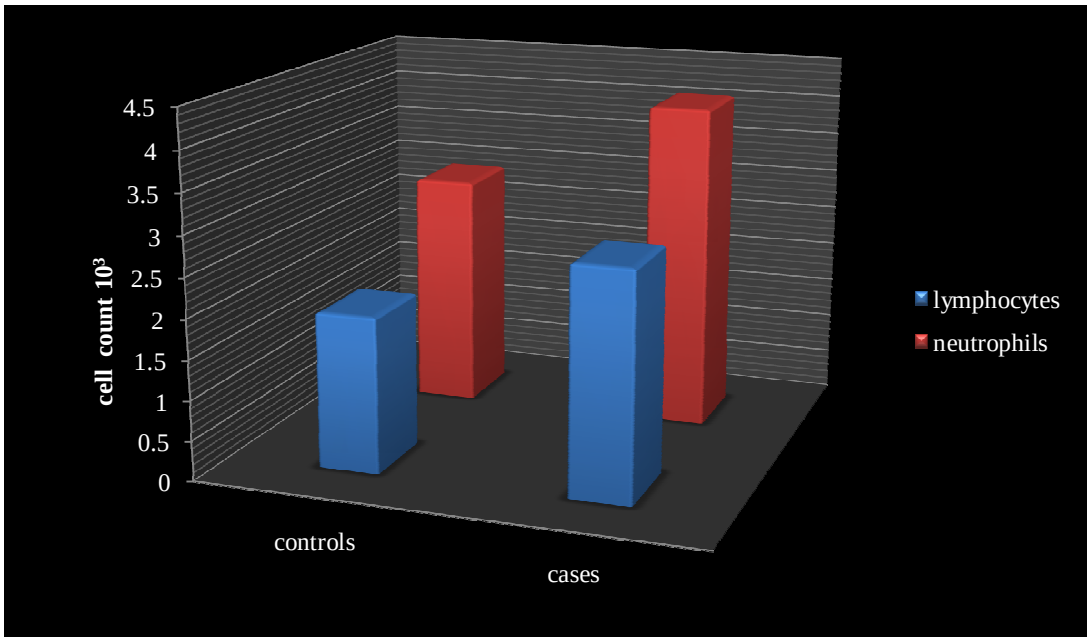


FIGURE III: COMPARISON OF PLATELETS COUNTS IN CONTROL &STUDY GROUP

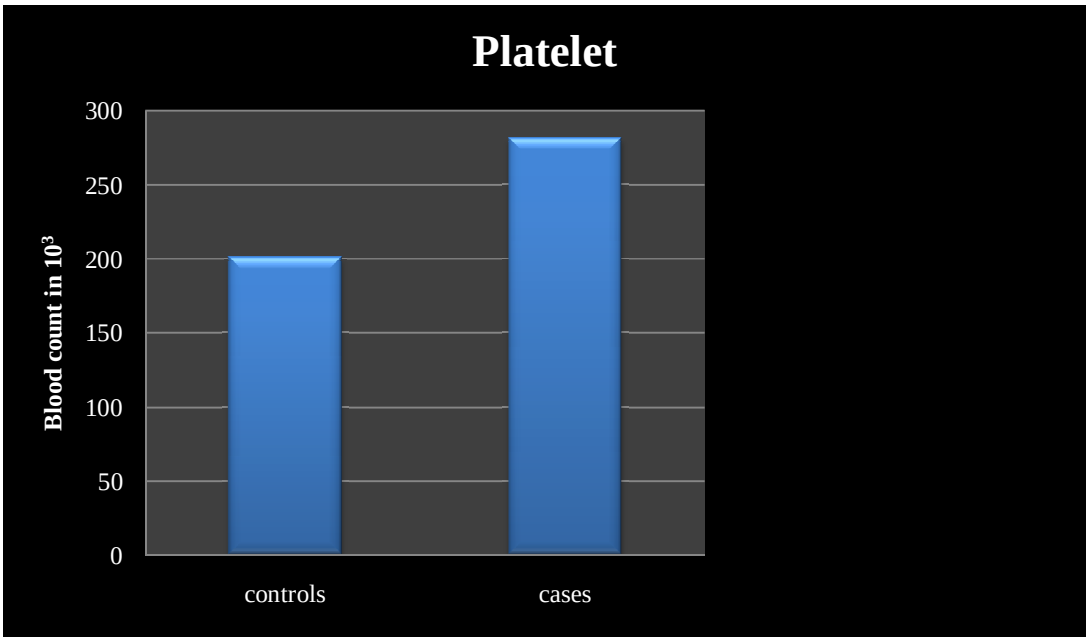


FIGURE IV: COMPARISON OF P- SELECTIN IN CONTROL AND STUDY GROUP

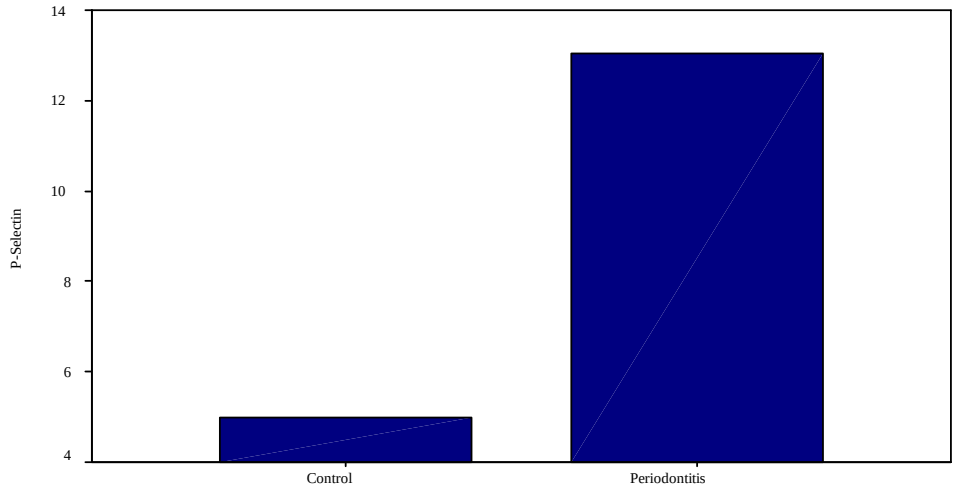


FIGURE V: COMPARISON OF SEVERITY OF PERIODONTITIS WITH P- SELECTIN EXPRESSION

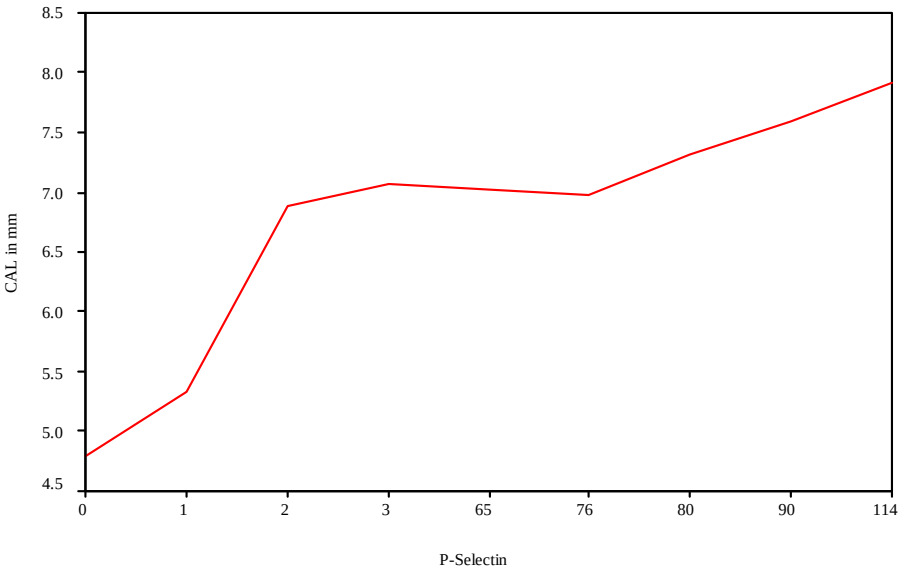


FIGURE VI: MORPHOLOGICAL CHANGES IN CONTROL GROUP

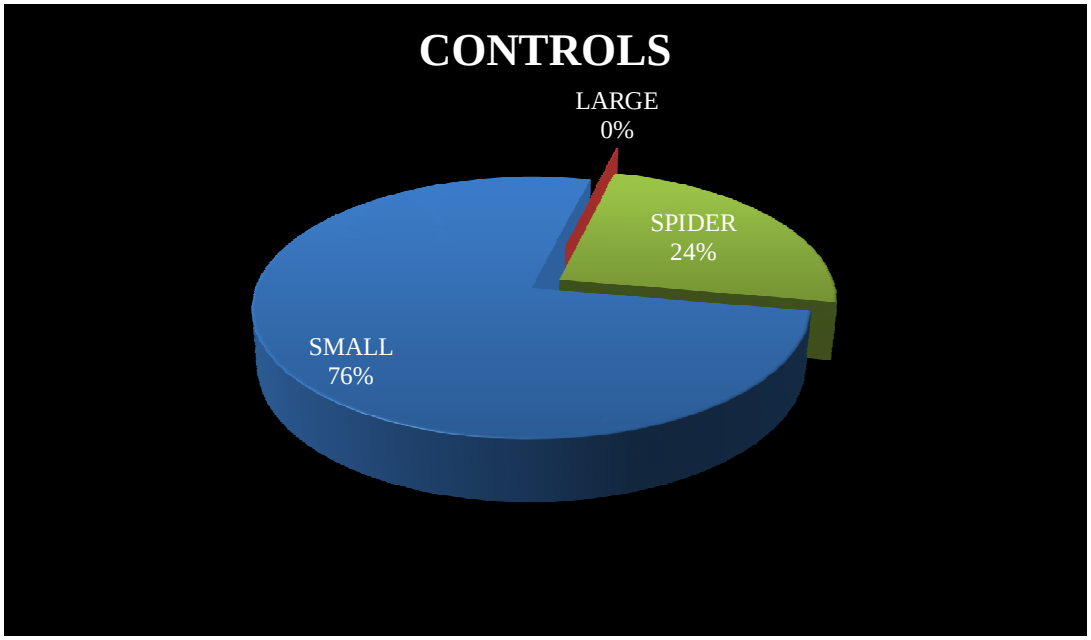


FIGURE VII : MORPHOLOGICAL CHANGES IN STUDY GROUP

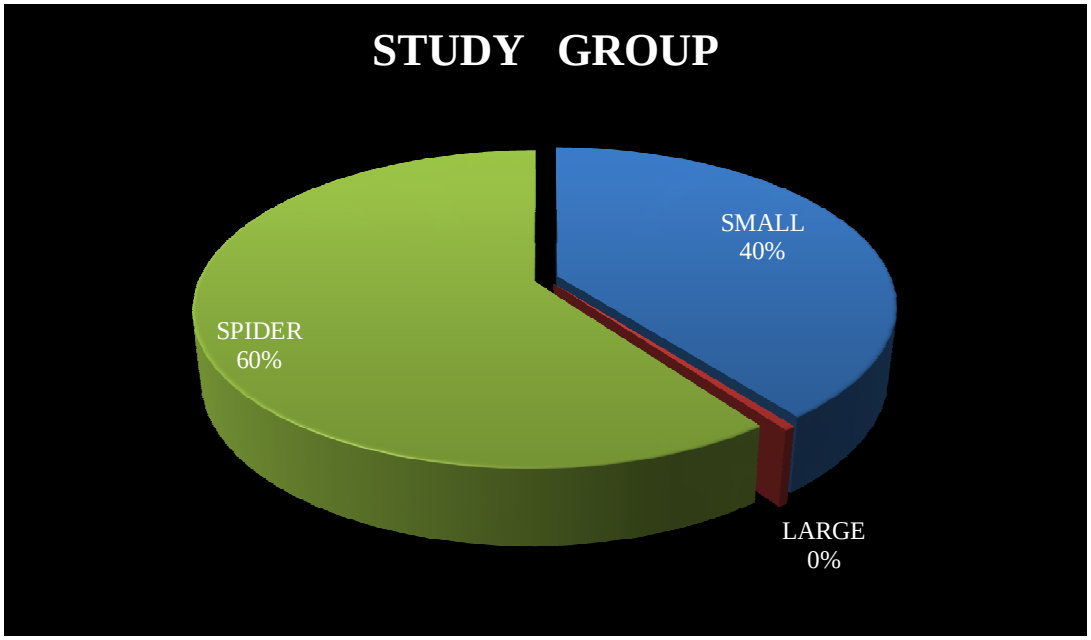


FIGURE VIII : PLATELET AGGREGATION GRADING DISTRIBUTION IN CONTROL AND STUDY GROUP.

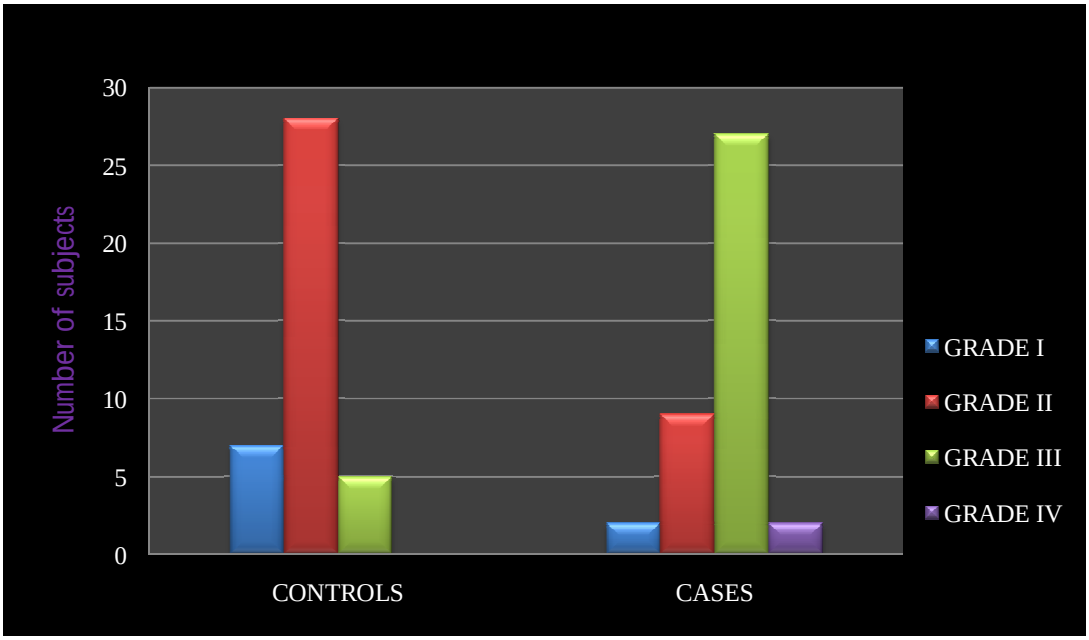
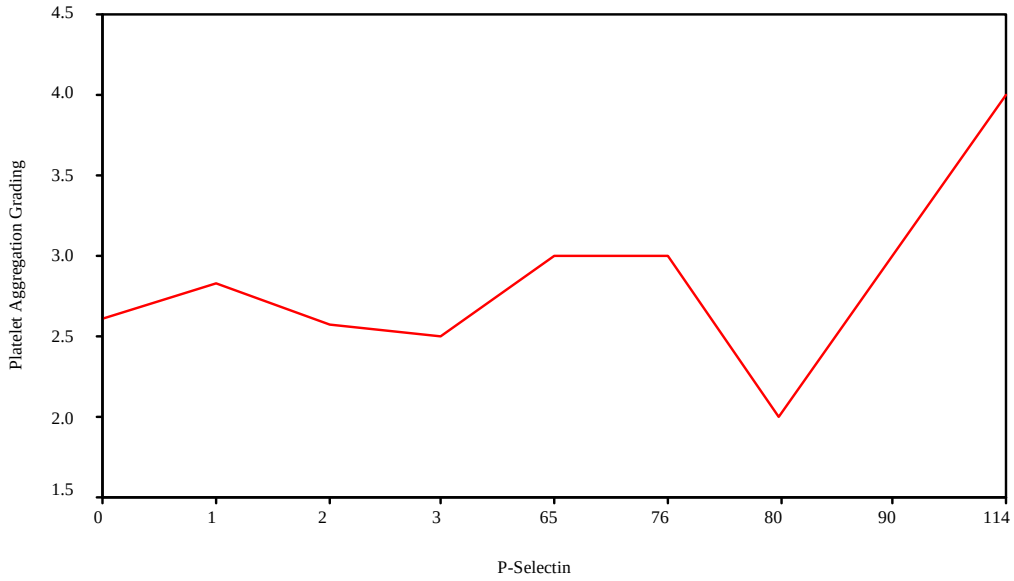
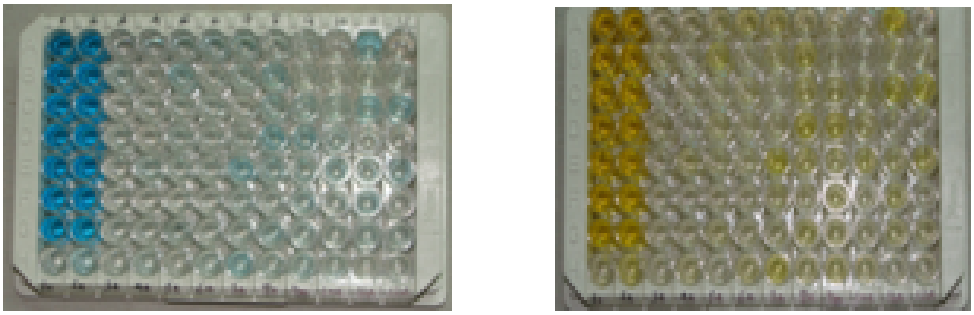
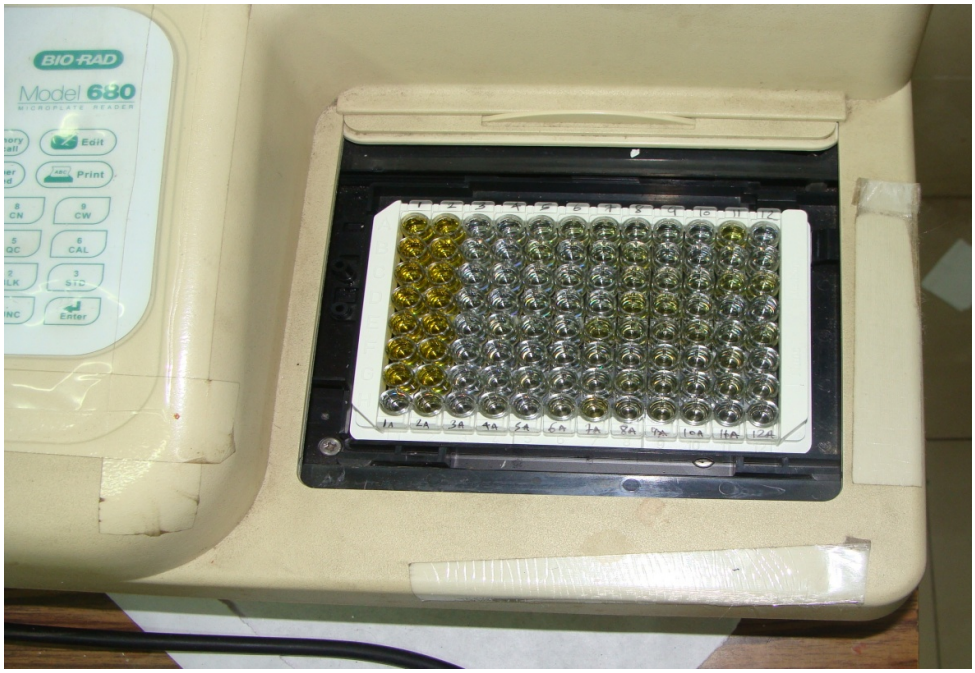


FIGURE IX: CORRELATION OF P-SELECTIN AND PLATELET AGGREGATION

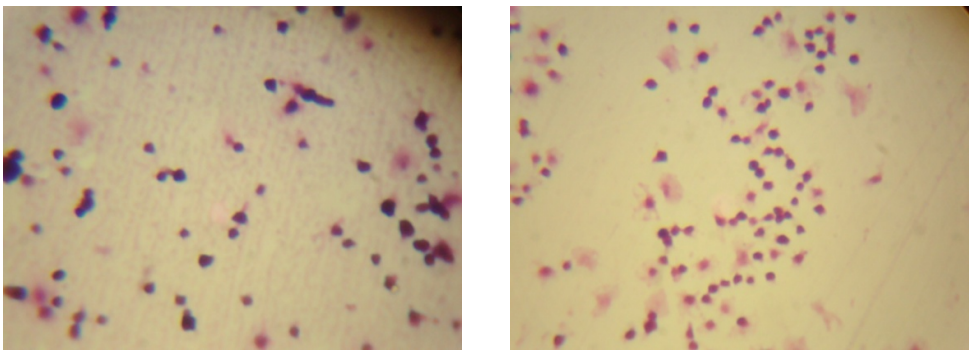




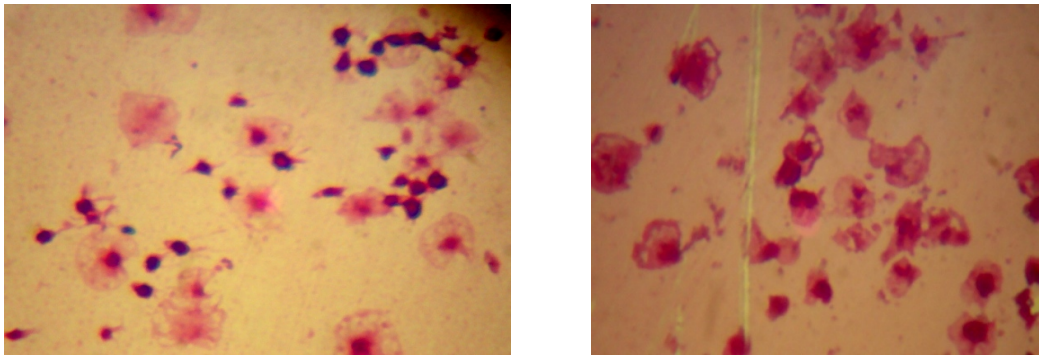
Photograph No :13 shows microplate wells during and after ELISA reaction



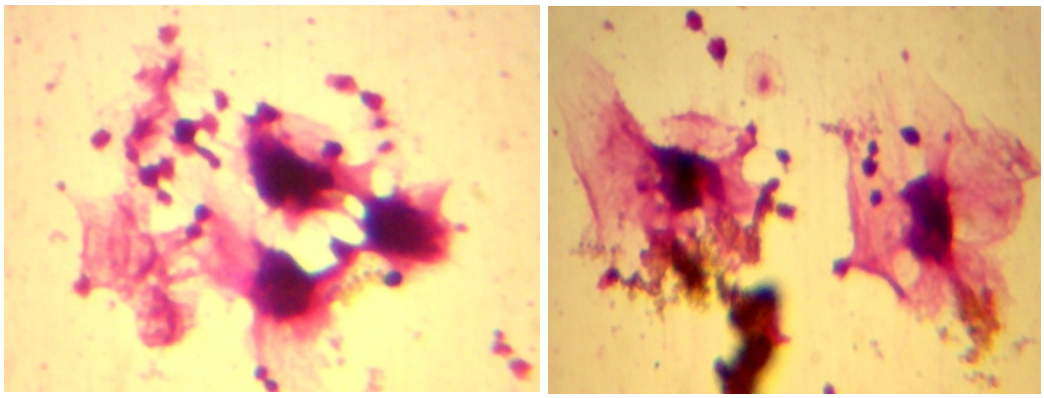
Photograph No :14 shows microplate wells on ELISA reader



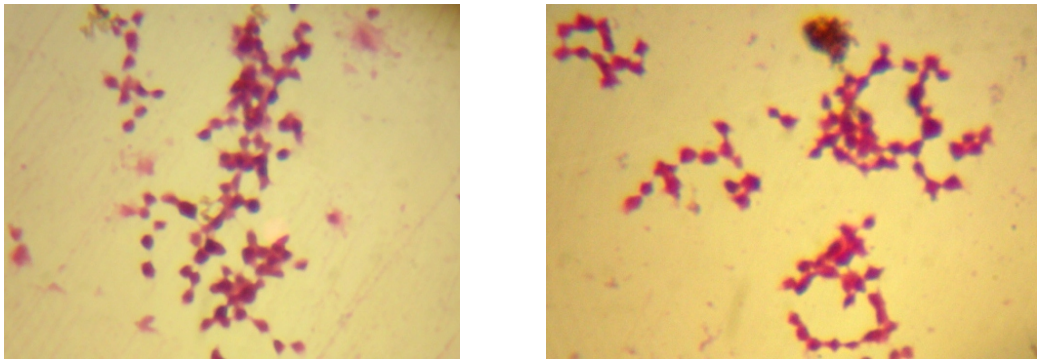
Photograph No :15 shows small and spider form platelets in control group



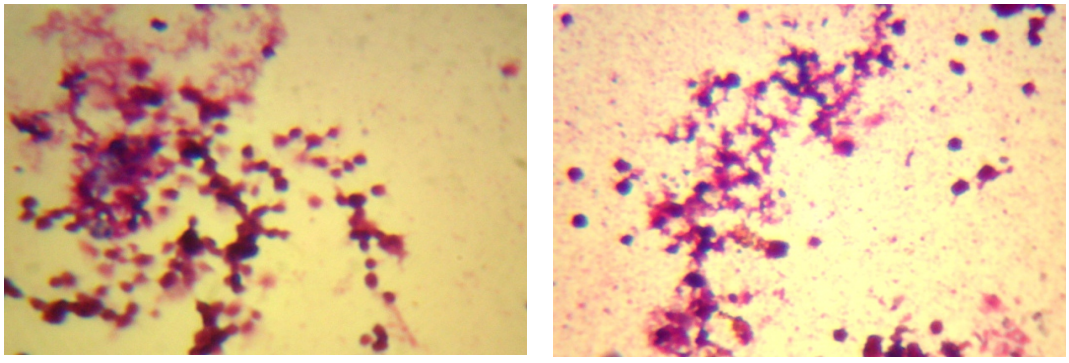
Photograph No :16 shows Spider forms of platelets in study group



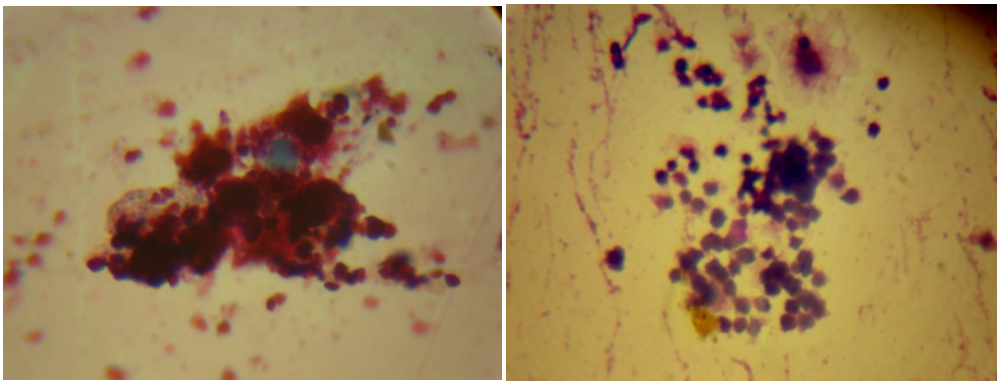
Photograph No :17 shows higher magnification of of Spider forms platelets



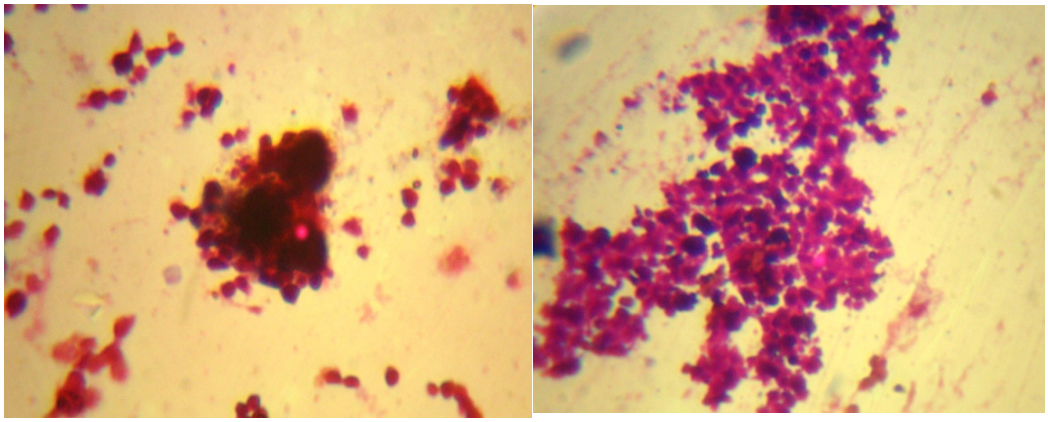
Photograph No :18 Reversible platelet aggregation (physiological aggregation)



Photograph No :19 Irreversible platelet aggregetion with ruptured membrane (secretion reaction)



Photograph No :20 Irreversible aggregation pattern (pathological aggregation)



Photograph No :21 Larger irreversible aggregation pattern in study group

DISCUSSION

Periodontal disease may be related to a number of systemic diseases including increased incidence of atherosclerosis, coronary heart diseases, stroke,diabetes mellitus, pre-term low birth weight delivery and respiratory diseases.***(Mealey BL 1999)***[75]**.**

Recently,the relationship between periodontitis and cardiovascular diseases is getting much attention particularly towards the role of putative periodontal pathogens like,P.gingivalis,A.actinomycetemcomitans. Either local inflammatory stimuli (leaking from infected periodontal tissues into circulation) or systemic inflammatory stimuli (resulting from immune responses to periodontitis-associated bacteremia) can induce activation of endothelial cells and/or platelets. Furthermore, direct interaction between pathogens and endothelial cells and/or platelets might contribute to the development of cardiovascular disease ***(Li L, Messas E,2002.)***[65]

Platelet activation has been implicated in the pathogenesis of a number of diseases, which including atherosclerosis**,** coronary vascular disease, and cerebrovascular disease***(Cahill MR,1993)***[23]. Abnormal platelet activation has also been associated with atrial fibrillation ***(Minamino,1999)*** [78]**,** cancer, peripheral vascular disease, Alzheimer disease ***(Sevush S,1998)***[101] , inflammatory bowel disorders ***(Collins CE,1994)***[25] and deep vein thrombosis ***(De Boer AC,1981)***[30] . Platelet activation seems to be influenced by diabetes mellitus, smoking ,hypertension and also the use of oral contraceptives ***(Kamath.S 2001).*** [94]

Platelet activation comprises a change in platelet shape, platelet aggregation and the release of platelet constituents. According to ***Kamath.S 2001***[94] , no single test

for the quantification of platelet activation is perfect and each has its own merits and demerits.

In the present study, platelet activation was evaluated by two methods. A quantitative analysis was done in order to estimate the serum sP – selectin level by ELISA method and a qualitative analysis was done to evaluate the morphological changes and aggregation pattern of platelets by spreading analysis test using light microscopy in both study and control group.

All the subjects were thoroughly examined and medical history was recorded. Subjects with systemic diseases like Diabetes mellitus, hypertension, cardiovascular disease, stroke, systemic lupus erythematous , and habits like smoking, tobacco, cocaine abuse were excluded.***(Blann 1997)***[11]

Subjects under medications like Aspirin, NSAIDS, Ticlopidine, Clopidogrel, Moxalactam, Losartan, Procaine, Dibucaine, Mithramycin, Diphenhydramine, Chlorpheniramine were excluded as they cause impairment of platelet aggregation. ***(Anjali& Shaprio et al 2008).***[3]

Also subjects with history of trauma or tooth extraction 2 weeks prior to the study and pregnant women were excluded from the study.

For blood sample collection, 20G needle was used . Because the use of needle with narrow diameter may activate the platelets due to shear stress against its wall.

Fasting venous blood sample was collected from both study and control groups because some of the food contents may activate platelets. Foods like Omega-3 fatty acids, fish oil , onion, cumin, turmeric, clove decrease platelet thromboxane production thereby may inhibit the platelet aggregation. Vitamin E, onion may induce inhibition of arachidonic acid metabolism and garlic inhibit the fibrinogen binding to platelets and impairs the platelet aggregation.

In the present study the mean age group for control group is 42.43 years and for study group is 43.20 years. The mean age of the study and control groups were similar.(TABLE III)

The gender distribution of the study group was Males – 19, Females – 21 and control Males – 17 ; Females – 23. In the present study the gender distribution between both the study and control group was similar. (TABLE IV)

The Mean PPD value of control group is 2.11mm and of the study group is about 4.9mm and the Mean CAL value of study group is 5.8mm (TABLE V).

According to AAP classification 1999 the severity of Periodontitis is divided into 3 groups.

i) Mild periodontitis - CAL 1-2mm

ii) Moderate periodontitis - CAL 3-4mm

iii) Severe periodontitis - CAL > 5mm

An elevated WBC count(reactive leucocytosis) in whole blood analysis was observed among the study group(8.04± 1.84) in contrast to the control group (5.46±1.84) with statistical significance of $P<0.001$ (TABLES VI & FIGURE I) .

Fredriksson 1998 [37] **& *Loos et al 2000*** [68] found significantly higher leukocyte count in patients with periodontitis. The leukocyte count has been demonstrated in several epidemiological studies to be an independent predictor of future coronary heart diseases **.**

Considering the differential blood count , the neutrophil count is higher in study group (control 2.94 ± 0.68 & study group 4.09 ± 1.50) . Lymphocyte count is elevated (control 1.95 ± 0.55 & study group 2.83 ± 0.72) among the study group and is found to be statistically significant (P<0.001) (TABLES VII & FIGURE II)

Also data reveals an elevated RBC count (control 3.99 ± 0.58 study group 4.41 ± 0.51) with P-value 0.001 and Hb% (control 11.43 ± 1.32 gm % study group 12.20 ± 1.87 gm% with P-value 0.035) among the study group which is statistically significant.

Reactive thrombocytosis was observed in study group compared to control group. The mean Platelet count in the study group was 281.25 ± 84.5 and in the control group 200.98 ± 56.9 which is found to be statistically highly significant (P=<0.001). This finding concurs with study of ***Wakai et al 1999*** [111]who stated significant association between platelet counts and periodontitis and systemic inflammation causes an increase in the number of platelets and platelet activation***.(Klinger 2002.)***[63]

Most stimuli cause changes in shape of platelets. This change involves first the formation of very fine (0.1 μm diameter) pseudopodia (i.e., filopodia) from the

rim of the disc, followed by a general "rounding up" of the platelet so that it becomes a spiny sphere, often with much broader pseudopodia.*(Nachmlas VT.1983)*[81]

The fine filopodia are formed of tight bundles of actin filaments when they are demembranated with nonionic detergent and examined by negative staining. According to ***Nachmlas VT,1977*** [80] when decorated with heavy meromyosin, they develop the well known "arrowheads" that point toward the cell body . This finding suggests that the filopodia are probably not "slid out" from the platelet by interacting with myosin, which would require the opposite polarity.

The second step in shape change is the change from a disc into an irregular sphere, is correlated with the phosphorylation of platelet myosin*(Daniel JL, 1984)*[29]**.** When platelets spread on surfaces, they first put out short filopodia and then the cytoplasm fills the space between them with what appears to be a network of actin filaments. Change in shape with or without secretion causes the microtubule bundle that lies beneath the rim of the disk to become centralized and surround the platelet granules, which are consequently concentrated toward the center of the platelet *(White JG.1974).* [114]

Platelet shape change can be measured in vitro under conditions that probably mimic in vivo platelet shape change by flow cytometry or electron microscopy ***(Holmsen H.1994)***[50]. Shape change is seen in an aggregometer tracing as a decrease in light transmission and loss of the oscillation. If aggregation follows, this decrease is succeeded by increased light transmission.

In the present study the platelet shape changes are evaluated by light microscopy using oil immersion objective with magnification of X 100 using plastic slides instead of glass slides. Because glass slides activate platelets in vitro (***Salzman 1963).***[96] The morphological changes of platelets in light microscopy is

followed as per the classification given by ***Scharrer 1985***.[97] Among the five types 3 forms like small, big & spider forms are more prevalent and other forms was very rare . So in present study small, big, & spider forms were considered for percentage calculation.

Small forms were higher in control group is about 75.83% compared to the study group 39.08 % . There was no significant difference in the big forms between the study and control group. Study group showed a higher percentage of spider forms (59.32%) while in the control group it is 23.88% . The observation showed high statistical significance with P- value <0.001 for small and spider forms and no statistical significance for the big forms P =0.075. Spider form is considered as a activated form of platelets. This indicates that platelet activation is higher among the study group compared to the control group.(TABLE X & FIGURE VI and VII : Photograph No. 15,16,17)

Higher percentage value of spider forms in study group may infer that stimulation of platelets due to the persistent periodontal bacterial toxins causes activation of platelets and their shape changes . Compared to electron microscope, light microscope do not faithfully register local differences in their thickness, but do accurately show changes in their length and orientation. .

Several studies have demonstrated that putative periodontal pathogen P. Gingivalis can activate blood platelets and induce platelets aggregation using platelet aggregometry method. Gingipains (Rgp and Kgp) from P. Gingivalis was able to induce platelets aggregation ***(Curtis 1993)***[27] and this result is in agreement with ***Sharma et al 2000*** [102] that P. Gingivalis vesicles posses potent platelet aggregation activity due to active concentration of many virulence factors.

P. gingivalis-induced platelet aggregation in platelet rich plasma (PRP) depends on hemagglutinin A (HagA)-encoding genes that intragenically code for adhesions, such as hemagglutinin domain proteins Hgp44, but not Arg-gingipain (Rgp) proteinase. ***(Naito 2006)*** [82].

Keber 1979 [61] evaluated the anti- aggregating property of propananol based on ***Breddin 1965*** [14]classification on platelet aggregation. The present study is also according to the classification given by ***Breddin 1965***[14] light microscopy. Observation revealed , Grade I & II aggregation patterns were more in control group whereas Grade III pattern was higher in the study group . Grade IV aggregation is seen only in the study group. None of the group showed grade V aggregation pattern. (TABLES XI & FIGURE VIII)

According to ***Breddin's*** [14]classification grade I & grade II is considered as a physiological aggregation(reversible aggregation) and grade III & IV is considered as a pathological aggregation Irreversible aggregation) (See photograph No.18- 21) Result of the present study showed higher percentage prevalence for pathological aggregation in the study group (grade III 67.5% grade IV 5%) compared to the control group (grade III 12.5 % and grade IV 0%).

The limitations of spreading analysis test is that it cannot define the aggregation size like platelet aggregometry . In platelet aggregometry , the aggregation can be classified as small (diameter 9-25 μm), medium (diameter 26-50 μm), and large-sized aggregates (diameter >50 μm) based on their size ***(Ozaki Y, 1994)*** [86]**.** However spreading analysis test shows the grade of platelet aggregation and helps us to decide the pathological aggregation .

Several studies have proved that activated platelets expressed increased p-selectin level which has a vital role in

atherosclerotic lesion development.***(Burger Peter C 2003)***[21]**.** Raised levels of soluble P-selectin are found in a variety of thrombotic disorders, including ischaemic heart disease **(*Ikeda H,1994)***[51] , cerebrovascular disease, congestive cardiac failure ***(O'Connor CM,1999)***[84] , and hypertension .***(Lip GYH, 1995)***[66]

Measurement of plasma levels of beta thromboglobulin and Platelet Factor 4 are specific to platelet release ***(Schernthaner G,1979)*** [98] and has been suggested as a means for detecting increased platelet activation in-vivo ***(Kaplan KL,1981)*** [59]**.** However some problems are evident with respect to measurement of these proteins. Thromboglobulin and Platelet Factor 4 are theoretically present in platelets in similar amounts and are released in similar quantities, but plasma levels of beta thromboglobulin greatly exceed plasma levels of Platelet Factor 4. This is possibly due to the more rapid binding of Platelet Factor 4 to the endothelial cells .Therefore a higher ratio of beta thromboglobulin to Platelet Factor 4 is always maintained in vivo. ***(Kaplan KL1981)***[59]. Due to above mentioned limitations we have P-selectin..

Moreover , activation of α-granules need higher threshold value compared to GPIIb/IIIa which need lower thershold value. ***(Michael .B.Holmes 1999)***[76] and hence the influence of extraneous factors like speed of centrifugation , temperature can be eliminated.

Michelson et al 1999 [77] study showed that activated platelets shed their membrane bound P-selectin in the plasma. ***Papapannagiotou et al 2009*** [88] was the first to document increased plasma levels of sP-selectin in chronic periodontitis. Result of the study showed that sP-selectin was statistically highly significant when compared with control group and positively associated with severity of periodontitis. According to this study increased level of P-selectin expression is from platelets and not

from endothelium, as vWf (marker of endothelial activation) was normal in their study.

Assinger.A 2010 [4] (Unpublished data) also documented the increased level of P-selectin associated with periodontitis and correlated with the severity of the disease. According to this study both platelets and endothelial cells seem to be responsible for the observed increased in plasma levels of soluble P-selectin in periodontitis. However, since platelet derived P-selectin is identical to P- selectin derived from endothelial cells ,the cell type specific contribution was not known.

In our study, sP-selectin expression is significantly higher in the study group when compared with control. The mean value for control group is 4.97 ± 16.56 ng/ml and study group is 13.05 ± 29.94 ng/ml is found to be highly significant (P<0.001) .(TABLE VIII & FIGURE IV)

Level of P-selectin expression is higher in severe periodontitis (CAL >5mm) compared to moderate periodontitis (CAL< 5mm).(TABLE IX & FIGURE V) Results of this study concurs with the reports of ***Papapannagiotou 2009***[88] and ***Assinger.A 2010*** [4]. However we have not evaluated the source of P-selectin

In the present study, 3 of the healthy controls had elevated soluble P-selectin levels even when their pocket depth was ≤2 mm. ***Assinger.A 2010*** [4] also documented similar findings in his control group and revealed the presence of *A.actinomycetemcomitans Y4 or P. gingivalis* among those controls.

The influence of P-selectin on platelet aggregation was correlated with Pearson's correlation coefficient test which showed a positive correlation (P- value = 0.005). (TABLE XII &FIGURE IX). According to ***Micheal et al 1999***[76] P-selectin determines the size and stability of platelet aggregation. P-selectin, which is

progressively expressed on the platelet surface, binds by means of the lectin domain to its binding site on adjacent platelets, stabilizing interactions between already-bridged platelets, thereby allowing the formation of large stable platelet aggregates**.** ***(Boukerche H 1996)***[13]

Moreover , the role of P-Selectin in atherosclerotic lesion development is very important. According to ***Rinder 1991*** [93], P-selectin expressed by platelet activation causes the formation of platelet-neutrophil and platelet-monocyte aggregates in blood . ***Berger P. C. 2003*** [21] states that P-selectin on microparticles could coat monocytes and contribute to their recruitment into atherosclerotic lesions.

Study done in P-selectin deficient mice have proved that both atheroselerotic lesion formation **(*Johnson RC 1997*** [56]**, *Collins RG 2000)***[26] and neointimal growth upon arterial injury were attenuated in apoE (apoE-/-) & LDL –receptor (LDLR-/-) deficient mice.

In the present study , both quantitative (estimation of serum P- selectin level) and qualitative analysis(morphological changes and pathological aggregation pattern) findings are positively correlated with chronic periodontitis and their disease severity. Thereby we infer that periodontitis associated with platelet activation and disease severity may aggravate the further platelet activation.

SUMMARY & CONCLUSION

Eighty subject were included in this study. The subject were catergorized into two groups. Cases (n=40) and controls (n=40). Thorough history was taken and the periodontal parameters like plaque index, bleeding index, probing depth, CAL were recorded. Blood samples were collected and platelet activation assessed by estimation of serum sP-selectin level by ELISA method and morphological changes of platelets and their aggregation pattern were evaluated by spreading analysis test using light microscopy.

The results of the study showed ,higher number of spider forms and significant pathological aggregation pattern in study group which indicates activation of platelets . An elevated serum sP-selectin level was seen in periotontitis patients compared to the control. Also sP-selectin expression increased in severe periodontitis than moderate periodontitis. Number of platelets, total WBC, lymphocytes, neutrophils, were increased in study group than control group.

The higher incidence of bacteremias and intravascular dissemination of bacterial products and inflammatory cytokines in periodontitis subjects could possibily provide an explanation for the current findings.***(Forner L, 2006)***[36] **.** These products have the potential to activate platelets which in turn can release an arsenal of potent inflammatory and mitogenic substances leading to an altered endothelial function (chemotaxis and adhesion). Thus a procoagulant state is established which provides a possible explanation for the increased risk for coronary heart disease in periodontitis subjects compared to controls as proved by epidemiological observation of ***Janket SJ 2003*** [53].

Chronic periodontitis induces systemic inflammation which ultimately leads to activation of platelets and translocation of sP-selectin from α – granules. This might be a cause for the morphological changes of platelets and occurrence of the increased percentage of activated form (spider form) .The pathological aggregation of platelets demonstrated in chronic periodontitis patients could be possibly due to elevated serum sP-selectin level induced by systemic inflammation.

Platelets play a central role in the process of thrombus formation (thrombogenesis***) (Hoak JC.1988)*** [47], as well as an important role in atherogenesis ***(Rabbani LE, 1994)*** [92] and the progression of atherosclerotic lesions. The interaction of the platelet with the vessel wall and its subsequent contribution to atheroma formation and thrombosis is of pivotal importance in the aetiology and pathogenesis of peripheral, coronary, cerebrovascular and other vascular diseases ***(White JG 1994).***[114]

Results of the present study shows an increased expression of P-selectin , spider form of platelets and pathological aggregation pattern which indicates that platelet activation may be associated with chronic periodontitis though the exact mechanism could not be established . Chronic periodontitis leads to platelet activation it may be emphasized that periodontitis can be an contributing factor in the development of cardiovascular disease .

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APPENDIX

PROFORMA

EVALUATION OF PLATELET ACTIVATION BY ESTIMATING SERUM sP-SELECTIN LEVEL AND MORPHOLOGICAL CHANGES OF PLATELETS IN PERIODONTAL HEALTH AND DISEASE.

Name : Age / Sex :

O.P. No : Code No :

Occupation : Income :

Address and Contact No.:

Chief Complaints

Pain / Shaky teeth / Bleeding gums / Swollen Gums / Receding Gums / Pus Discharge / Increase in Spacing between teeth / Stains / Others.

Duration:

Medical history

1. Trauma or tooth extraction
2. Patients drug history(antibiotics, anticoagulants)
3. Any chronic infectious diseases
4. Pregnancy
5. Anemia, bleeding disorders or any other hematological disorder
6. Cardiac diseases
7. Stroke

Dental history

Periodontal treatment within past 6 months.

Clinical Examination

GINGIVAL BLEEDING INDEX – AINAMO & BAY (1975)

18	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

PLAQUE INDEX – SILNESS & LOE (1964)

18	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

PROBING DEPTH (PD) & CLINICAL ATTACHMENT LEVEL (CAL) (in mm)

MAXILLARY:

Palatal

CAL																
PPD																
	18	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
PPD																
CAL																

Buccal

MANDIBULAR:

Lingual

CAL																
PPD																
	48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
PPD																
CAL																

Buccal

DIAGNOSIS:

INVESTIGATION:

Morphological changes **- small =**

- Big =

- spider =

Platelet aggregation **-**

sP- Selectin **-**

Platelets number **-**

INFORMED CONSENT FORM

STUDY TITLE**:**

EVALUATION OF PLATELET ACTIVATION BY ESTIMATING SERUM sP-SELECTIN LEVEL AND MORPHOLOGICAL CHANGES OF PLATELETS IN PERIODONTAL HEALTH AND DISEASE.

Name: O.P.No:

Address: Code No:

Tel. no:

I,_____age____years exercising my free power of choice, hereby give my consent to be included as a participant in the study "**Evaluation of platelet activation by estimating serum sP-selectin level and morphological changes of platelets in periodontal health and disease"**

I agree to the following:

- I have been informed to my satisfaction about the purpose of the study and study procedures including investigations to monitor and safeguard my body function.
- I understand that the lab investigations will require the procurement of my blood in required amount.
- I agree to cooperate fully and to inform my doctor immediately if I suffer any unusual symptom.
- I have informed the doctor about all medications I have taken in the recent past and those I am currently taking.
- I hereby give permission to use my medical records for research purpose. I am told that the investigating doctor and institution will keep my identity confidential.

Name of the patient Signature / Thumb impression

Name of the investigator Signature

ஆராய்ச்சி ஒப்புதல் படிவம்

ஆராய்ச்சி தலைப்பு

“இரத்த தட்டு செல்களின் தூண்டுதலை குருதி பாய்ம திரவத்தில் கரையும் தன்மையுள்ள பி-செலக்டின் அளவினையும் மற்றும் தட்டு செல்களின் உருவ வேறுபாட்டையும் பற்புறத்திகக்களின் நோயுற்ற மற்றும் நோயற்ற நிலையில் ஆராய்ந்தறியும் ஓர் ஆய்வு”.

தேதி : புறநோயாளி எண் :

பெயர் : ஆராய்ச்சி சேர்க்கை எண் :

முகவரி : வயது : ஆ/பெ :

தொலைபேசி எண் :

நான் _____ வயது _____ என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக் கொள்ள சம்மதிக்கிறேன்.

எனக்கு விளக்கப்பட்ட விஷயங்களுக்கு நான் எனது சம்மதத்தை தருகிறேன்

❖ இந்த ஆராய்ச்சியின் நோக்கம் மருத்து முறைகள் பரிசோதனை முறைகள் எனக்கு திருப்தியுறும் வகையில் விளக்கப்பட்டன.

❖ நோயின் தன்மை அறியும் செயலின் ஒரு பகுதியாக என் உடலில் இருந்து சிறிதளவு இரத்தம் எடுக்கப்படும் எனவும், அது எந்த விதத்திலும் சிகிச்சையும் என் உடல் நலத்தையும் பாதிக்காது என்பதையும் அறிந்துக் கொண்டேன். மேலும் வழக்கமாக செய்யும் இரத்த பரிசோதனையும் இந்த ஆய்வுக்கு உபயோகப்படுத்தப்படுகிறது என்பதை உணர்ந்துக் கொண்டேன்.

❖ எனது மருத்துவ குறிப்பேடுகளை இந்த ஆராய்ச்சியில் பயன்படுத்திக் கொள்ள சம்மதிக்கிறேன். ஆராய்ச்சி மையமும் ஆராய்ச்சியாளரும் என்னுடைய பெயர் மற்றும் சில விபரங்களை இரகசியமாக வைப்பதாக அறிகின்றேன்.

பேராசிரியரின் கையொப்பம்	ஆராய்ச்சியாளரின் கையொப்பம்	நோயாளியின் கையொப்பம்